

Klinik für Kleintierchirurgie der Veterinärmedizinischen Fakultät  
der Universität Zürich

(Direktor: Prof. Dr. P. M. Montavon)

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Unter der Leitung von Dr. S. Tepic

# **Inhibition of endogenous sources of arginine in arginine depleted dogs**

INAUGURAL-DISSERTATION

zur Erlangung der Doktorwürde  
der Vetsuisse-Fakultät  
Der Universität Zürich

Vorgelegt von

Goran Cvetkovic

Tierarzt

von Smederevo, Serbien

Genehmigt auf Antrag von

Prof.Dr.med.vet. P. M. Montavon, Referent  
Prof.Dr.med. Christopher H. Evans, Korreferent

Zürich 2009

Zentralstelle der Studentenschaft

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## ABSTRACT

Cancer is one of the most serious unmet medical needs both for humans and pet animals. Cancer is not a disease, is rather a diverse group of diseases, which scientists struggle to understand for centuries. Complex, still undeciphered etiology, a series of clinical signs, metastasing, recurrence and miserable therapy results put cancer on top of the research agenda of innumerable scientific institutions and individuals all around the world.

Arginine is one of the most metabolically versatile amino acids, which serves as a precursor for the synthesis of urea, nitric oxide, polyamines, proline, glutamate, creatine and agmatine. It seems that arginine also plays crucial metabolic roles in growth and dividing of the cancer cells. Several *in vitro* studies have proved the arginine auxotrophy of different cancer cell lines, notably hepatocellular carcinoma, melanoma, lymphoma and lung carcinoma. Without arginine and other urea cycle intermediates (ornithine and citrulline), most cancer cells die within days.

Besides its metabolic role complexity, arginine's semiessential or conditionally essential nature (due to *de novo* synthesis of arginine in mammals) builds a labyrinth of constraints to anyone who attempts to kill cancer *in vivo* by depleting arginine.

To deplete circulating arginine, one has to overcome powerful homeostatic mechanisms (diet, protein breakdown, *de novo* synthesis) that keep arginine circulating level in a constant range (about 100  $\mu\text{mol/l}$ ). Before the depletion is achieved, severe side effects should be anticipated and prevented with timely and accurate interventions in order to keep the cancer patient alive. Only if circulating urea amino acids (arginine, ornithine and citrulline) are depleted and maintained below 1  $\mu\text{mol/l}$  and all the potential side effects of this depletion are prevented, cancer can be killed *in vivo* and the patient can survive the treatment.

This thesis is a document about the experiments on 23 healthy beagle dogs at Vetsuisse Faculty University of Zurich, which goal was to deplete the circulating urea cycle amino acids below 1  $\mu\text{mol/l}$  for 24 hours.

Dogs were chosen rather than a lower species, as the tested treatment protocol is aimed to the cancerous dogs.

Understandably, potential favorable results could be leveraged for the treatment of humans with cancer.

The most successful variant of the tested protocol, which included arginase – for active degrading of arginine, insulin – for protein breakdown inhibition and lactate – for inhibition of proline oxidase, was able to deplete arginine and ornithine to below 5  $\mu\text{mol/l}$ .

However, none of tested protocol combinations was able to deplete arginine, ornithine and citrulline below the limit of detection (1  $\mu\text{mol/l}$ ).

Most disappointingly, none of the tested protocols had significantly depleted circulating level of citrulline.

An effective approach for depleting citrulline ought to be found.

## 1 INTRODUCTION

A treatment of an inoperable cancer has to be based on a leveragable difference between the cancer cells and the host cells. The difference must be so distinguishable that a treatment based on it, can kill the cancer but exempt the host cells.

The most vivid difference between the cancer and the host cells is an extraordinary growth and dividing intensity of the cancer cells. As the growth and dividing require proteins or amino acids, the cancer cells inevitably necessitate significantly more amino acids than the healthy ones.

During the last decades, there have been several attempts to exploit this different requirement in amino acids for an effective cancer therapy. The most successful example is the L-asparaginase as a part of a multi - drug protocol for treatment of childhood acute lymphocytic leukemia (ALL). The L-asparaginase hydrolyses asparagine to aspartic acid and ammonia, what inhibits protein synthesis in leukemia cells, which require exogenous asparagines.

Unfortunately, this L-asparaginase's effect is only transient as the ALL cells develop resistance to the asparaginase. It is therefore employed only for the induction of cancer remission.

L-arginine has also been target for a cancer treatment for over sixty years.<sup>1</sup> Arginase, an enzyme which hydrolyses arginine to ornithine and urea, and arginine deiminase, an enzyme which hydrolysis L-arginine to L-citrulline and ammonia, are the most successfully used enzymes in arginine depletion for cancer treatment.

There are at least three groups that currently work on arginine as a cancer therapy target.

The first group works at Pascale National Cancer Institute, Naples, Italy. It brought the PEGylated arginine deiminase (ADI-SS PEG 20,000 mw) to Phase I and II clinical trials for metastatic melanoma neoplasm in humans.<sup>2</sup>

The second one is led by Dr. Sci. S. Tepic, which carried out six rounds of in vivo experiments on arginine depletion, on mice and dogs, at Vetsuisse Faculty, University of Zurich, to establish the basic requirements for achieving significant systemic arginine depletion and to define the minimum number of pharmacological intervention necessary to control the various side effects of arginine depletion:

1. Round 1 (Andrea Maute, Ana Maurer, Michaela Messmer, Minder Marco, Pawel Pyk and Slobodan Tepic) did two series of experiments on dogs: extracorporeal arginine depletion means arginase in the dialysis filter and a selective dialysis. There was no significant plasma arginine reduction.
2. Round 2 (Andrea Maute, Nicole ----, Pawel Pyk, and Slobodan Tepic) tested inhibitory effect of insulin on dogs. Result: plasma arginine was 10  $\mu\text{mol/l}$ .
3. Round 3 (Pascale Binz, Linda Scott, DVM, Pawel Pyk, Slobodan Tepic) has figured out how to overcome lost of platelets if arginine is depleted.
4. Round 4 (Arnold Pierre, Marcus Vogelsanger, Pawel Pyk, Slobodan Tepic), treated dogs with liver extract and did liver cryo – ablation. Result: plasma arginine below detection.

5. Round 5 (Anja Gödl, Caroline Walder, Elaine Cambell, Slobodan Tepic). The aim was to kill cancer, in tumor – bearing mice, by depleting arginine with PEGylated arginase. No success. Tumor survived on citrulline.
6. Round 6 (Roman Camen, Ismet Hamza, Darek Mochnecki, Slobodan Tepic and Goran Cvetkovic), treated a cancerous dog with liver extract. Without success.

The third group is based in Hong Kong, led by Dr. Cheng, which used the work of the Tepic's group to produce PEGylated Arginase expressed in *B. subtilis*, and to bring this enzyme to Phase I/II clinical trial for liver cancer patients.<sup>3</sup>

Nevertheless, the promising opportunity for curing different types of cancer by arginine depletion has not been exploited yet. It seems that a more profound understanding of the homeostatic mechanisms involved in the arginine fate in the organism is needed.

This thesis is a document of a work in this direction.



## 2 PROJECT DESIGN

### 2.1 Arginine deprivation: principles and concepts

Since 1930, arginine has been known for its potential to influence growth of transplantable mice tumor.<sup>4</sup>

The first results of *in vitro* testing that proved damaging effect of arginine depletion to the cancer cells were published in 1960s.<sup>5</sup> Since then, a series of *in vitro* experiments have indicated that arginine depletion can be fatal for cancer cells.

However, following the evidence made by Tepic's group, Wheatley et al. have shown and published in 2005<sup>6</sup> that arginine depletion itself is not sufficient for damaging of all cancer types. They proved that there are cell lines that can survive without arginine, on citrulline +/- argininosuccinate.

In 2008, Wells et al have shown that leukemia cells (L1210) cannot proliferate in arginine free medium enriched with 10U/ml of bovine liver arginase.

Results of several *in vivo* experiments on mice<sup>7</sup> and dogs<sup>8</sup> were in alignment with the *in vitro* results. These results lead to a conclusion that most cancer cell lines will not be killed by arginine depletion unless the all other urea cycle amino acids (ornithine, citrulline and argininosuccinate) are depleted too. This conclusion seems logical as conversion of urea cycle amino acids within the urea cycle is an ongoing process in a live organism:

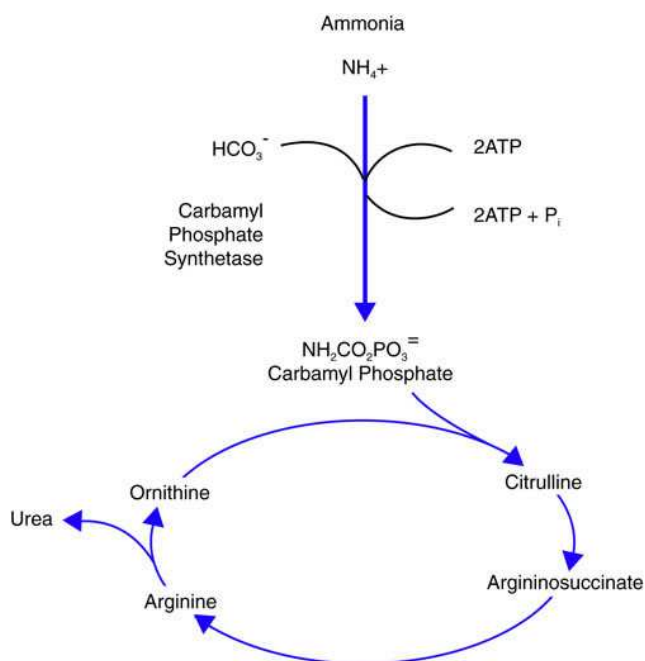


Figure 1: Urea Cycle

## 2.2 Aims of the study

The conclusions drawn from previous experiments at Vetsuisse Faculty University of Zurich<sup>9</sup> have defined the aims of this study, performed in 2002:

- 1) Defining the minimal number of pharmacological interventions necessary to deplete the urea cycle amino acids (arginine, ornithine, and citrulline).
- 2) Defining the minimal number of pharmacological interventions necessary to control the various side effects of arginine depletion.

## 3 MATERIALS AND METHODS

### 3.1 Experiments

#### 3.1.1 Procedure outline

The study was done on healthy beagle dogs. The experiments were approved by the authority (Kantonales Veterinäramt Zürich, No.68/2002). Dogs were preferred to mice, as the tested treatment protocol was planned for dogs with cancer. There are substantial differences between arginine, ornithine and citrulline metabolism in dogs and in lower species. In addition, for continuous measurement of arginine, ornithine and citrulline concentration in plasma, as well as levels of glucose and lactate in blood, 2 ml of blood every two hours were needed, which is impossible from a lower order species.

All planned experiments were conducted sequentially: there was only one dog in the experiment at a time. All dogs were in total anesthesia for the complete duration of the treatment protocol intervention. All these experiments were terminal – dogs were not awaked from anesthesia but euthanized after 24 hours. All sacrificed dogs did undergo pathology (standard) and histopathology (liver, kidney, gut, lungs, and heart) examination.

#### 3.1.2 Dogs

The study was done on 23 healthy beagle dogs, from which there were sixteen male and seven females. Dogs have originated from the University of Zurich, Aussenstation Stieghof, 8425 Oberembrach and from Hoffmann – La Roche AG, Basel. All the dogs had already been subjects of other experiments at Hoffman – La Roche AG or at School of Veterinary medicine Zurich and were planned for euthanasia. These previous experiments were done at least one month before the current study was started and had not compromised health of the animals, which was checked by clinical, hematological and biochemical examinations.

### 3.1.3 Treatment design

The treatment protocol was designed to inhibit all the arginine sources in the body: diet, protein breakdown in the muscles<sup>10</sup> and the renal - intestinal arginine synthesis pathway.<sup>11</sup>

The experimental dogs were divided into eight groups:

- 1) Control group
- 2) Insulin group
- 3) Proteasome inhibitors group
- 4) Insulin plus Proteasome inhibitors group
- 5) NSAID group
- 6) Dantrolene group
- 7) Lactate group
- 8) No arginase group

The groups did not have the same number of dogs, as the whole study was designed as a screen for the arginine inhibitory effect of the tested compounds, which were described in the literature to inhibit either protein breakdown or the arginine synthesis. The control group included four dogs. The number of dogs in the other groups depended on the effect of the tested compound on the arginine plasma level. As we did not see any expected effect in the group 3, 4, 5 and 6 we stopped further testing of these compounds, and devoted our resources to the groups 7 and 8.

<b><i>Control group</i></b>	<b><i>Insulin group</i></b>	<b><i>Proteasome Inhibitors Group</i></b>	<b><i>Insulin &amp; Proteasome Inh. group</i></b>	<b><i>NSAID group</i></b>	<b><i>Dantrolene group</i></b>	<b><i>Lactate Group</i></b>	<b><i>No arginase group</i></b>
Ringo, 14.8 kg  Tex, 11 kg  Tussi, 8 kg  Saly, 11.7 kg	Wako, 10 kg  Doris, 11 kg	Alf, 10 kg (Normosang, 25mg/ml)  Anton, 12 kg (Proferin ES, 6 Tablets)  Pluto, 15 kg (Proferin ES, 6 Tablets)	Leo, 11.3 kg (Insulin + Proferin ES, 6 Tablets)  John, 15 kg (Insulin + Proferin ES, 6 Tablets)	Tim, 16.8 kg	Rex, 12 kg	Ina, 11.2 kg (Na-lactate)  Sam, 14 kg (Na-lactate)  Arni, 12.2kg (Na-lactate, insulin)  Kim, 11 kg (Na-lactate, insulin, HCL)  Tina, 11 kg (DL MS, insulin)  Bob, 13 kg Maya, Ken, (Na-lactate/DL MS)	York, 14 kg  Ana, 13 kg

Table 1: The experiment groups

### 3.1.4 Treatment protocol

Except for group 8, the all dogs have obtained a 'control group drug protocol', which contained PEGylated arginase plus drugs that prevent arginine depletion side effects (see later). The group 8 did not receive PEGylated arginase. The all dogs, except from the control group, have received a group-specific testing compound in addition to the control group protocol.

### 3.1.5 Treatment administration

Most of the drugs of the tested protocols were administrated through an infusion system machine, produced by Fresenius Vial S.A. Le Grand Chemin F-3859 Brezins, France and delivered by Fresenius Kabi Schweiz AG, CH – 6371 Stans. The infusion system had administrated the combination of drugs through a central vein catheter.

The less number of drugs were given p/os or through a peripheral vein catheter.

### 3.1.6 Adverse events

Unfortunately, 6 dogs from the lactate group died during the experiment, as we did not anticipated correctly that the lactate bicarbonate and the lactic acid would have such fatal effects to the pH homeostasis of the treated dogs.

### 3.1.7 Experimental plan

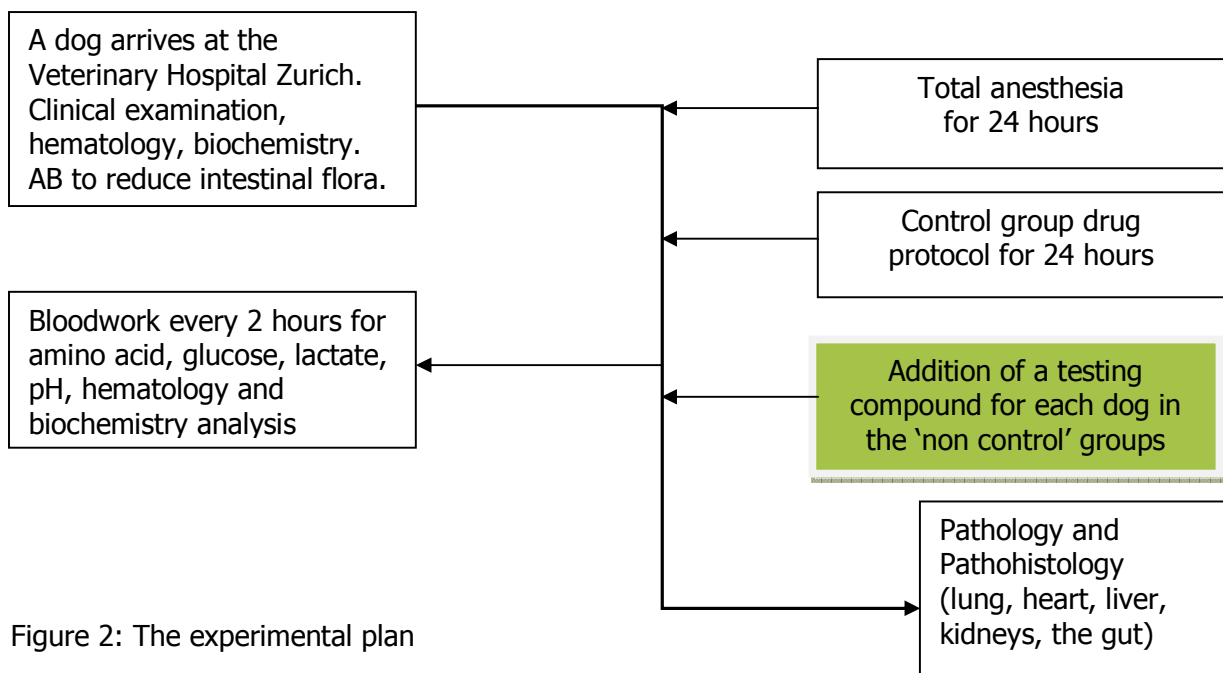


Figure 2: The experimental plan

### 3.1.8 The group treatments and expected outcome

<p>The control group has received i/v, with infusion system pumps:</p> <ol style="list-style-type: none"> <li>1) PEGylated arginase, activated with manganese. The role of arginase was to deplete circulating arginine.</li> <li>2) Sodium nitroprusside – SNP. Its role was to donor nitric oxide, a gas that suppresses platelet activation and intravascular thrombosis.</li> <li>3) Glypressin, was given to control the vasodilatory effect of the administered sodium nitroprusside.</li> <li>4) Sodium thiosulfate, given to neutralize cyanide, produced by administered sodium nitroprusside.</li> <li>5) Ilomedine, given to reduce platelets aggregation.</li> </ol>	<p>Expected outcome:</p> <p>In spite of given arginase, we expected the arginine, ornithine and citrulline plasma levels to be as in healthy dogs due to homeostatic mechanisms for maintaining arginine plasma level - protein breakdown and the arginine synthesis in the intestinal renal axis.</p>
<p>In addition to the 'control group protocol', the Insulin Group has obtained (i/v, infusion pump):</p> <ol style="list-style-type: none"> <li>1) Insulin, a potent protein breakdown inhibitor</li> <li>2) 50% glucose, to maintain normoglycemia, lowered by the given insulin</li> </ol>	<p>A lower arginine plasma level was expected than in the control dogs (30-40 <math>\mu\text{mol}</math> vs. 100 <math>\mu\text{mol}</math>) due to potent protein breakdown inhibitory effect of insulin.</p>
<p>In addition to the 'control group protocol', in the Proteasome Inhibitors Group two different proteasome inhibitors were tested:</p> <ol style="list-style-type: none"> <li>1) Normosang (i/v)</li> <li>2) Proferin ES (p/os)</li> </ol>	<p>A lower arginine plasma level was expected than in the control dogs due to protein breakdown inhibitory effect of proteasome inhibitors.</p>

<p>In addition to the 'control group protocol', in the Insulin plus Proteasome Inhibitors Group a synergic effect of these two compounds was tested. In addition to the 'control group protocol', this group get:</p> <ol style="list-style-type: none"> <li>1) Insulin (i/v)</li> <li>2) 50% glucose (i/v)</li> <li>3) Proferin ES (p/os)</li> </ol>	<p>A synergic inhibitory effect of insulin (lysosomal inhibitor) and Proferin ES (proteasomal inhibitor) was expected. A lower arginine plasma level than in the Insulin Group was expected.</p>
<p>In addition to the 'control group protocol', the NSAID Group has obtained:</p> <ol style="list-style-type: none"> <li>1) Metacam, a non-steroid anti-inflammatory drug to keep the cytokines, very potent protein breakdown stimulators, low.</li> </ol>	<p>A slight lower amino acid levels in plasma might have been expected.</p>
<p>In addition to the 'control group protocol', the Dantrolene Group has obtained:</p> <ol style="list-style-type: none"> <li>1) Dantrolene, a potent inhibitor of calcium release from the sarcoplasmic and endoplasmic reticula.</li> </ol>	<p>A lower arginine plasma level was expected than this in the control group.</p>
<p>In addition to the 'control group protocol', the Lactate group has obtained:</p> <ol style="list-style-type: none"> <li>1) Lactate in form of Sodium lactate or lactate acid. Lactate is known as a potent proline oxydase inhibitor, which is one of the enzymes that regulate production of citrulline in the enterocytes from proline, a non-essential amino acid.</li> </ol>	<p>A lower arginine, ornithine and citrulline plasma levels were expected, as lactate was known as a inhibitor of the intestinal renal axis of arginine synthesis.</p>
<p>In the Lactate without enzyme group only a source of lactate was given coupled with drugs for amelioration of arginine depletion side effects.</p>	<p>An arginine level higher than in the dogs with arginase was expected.</p>

Table 2: The expected outcomes of each experimental group

## 3.2 MEDICAMENTS AND CHEMICALS

### 3.2.1 Arginase

The arginase used in the present study was purified recombinant human liver arginase, expressed in *E. coli*, produced and delivered by Prof. M. Ikemoto at the College of Medical Technology in the University of Kyoto, Japan.

Prior to use, the arginase was heat-activated at 60°C for 10 minutes in presence of Manganese. In addition, arginase was PEGylated with cyanuric chloride-activated polyethylene glycol at pH 9.0 and room temperature for 30 minutes using the method described by Savoca et al. (1979). The PEGylation substantially prolongs plasma half-life of the arginase, which relatively low molecular weight in the native state of 35,000 Dalton allows a prompt glomerular filtration of the enzyme. Subsequently, the arginase was dialyzed against 5 liters of 1x phosphate buffer saline to remove any excess of PEG, using a HF80, Fresenius filter.

The enzyme was then sterilized by filtration through 0.22 µm Sarstedt filter and stored at 4°C pending use. During i/v administration, the bottle with the arginase solution was covered with ice-bags to protect the enzyme activity.

The arginase activity was 1500 Units per mg.

Dosage: 30 U/kg/day, via Orchestra Module MVP PT, infusion pump.

### 3.2.2 SNP

Sodium nitroprusside (Sigma) was given as a donor of Nitric Oxide (NO), whose concentration is low if arginine is deprived. The low NO concentration can lead to platelets activation and aggregation, loss of platelets and consequent bleeding.

An oral administration of SNP is impossible, as SNP is quickly inactivated in the GI tract. For i/v administration, 160 mg SNP was solved in the 10 ml NaCl, sterilized by filtration and then added NaCl to 100 ml. As SNP is light-sensitive, and breaks down in sunlight producing cyanide<sup>12</sup>, the drug preparation and administration has to be in absent of light. The infusion bottle and the infusion lines were covered with dark tape.

Dosage: SNP is given 2 mg/kg BW/day at the beginning up to 6 mg/kg BW/day, administrated via Orchestra Module DPS, infusion pump.

As SNP is a strong vasodilator and can release cyanide, Glypressin and Sodium Thiosulfate were administrated to ameliorate these side effects.

### 3.2.3 Glypressin®

Glypressin (Terlipressini Acetate, 1 mg), Ferring AB, Malmö, Sweden, was needed to correct a vasodilatation caused by SNP.

Two ampoules of 5 ml were diluted in 40 ml saline.



Dosage: 0.6 U/kg BW/day, administrated via Orchestra Module DPS.

#### 3.2.4 Sodium Thiosulfate

Sodium Thiosulfate (Sigma) is given as a donor of sulphur for the enzymatic building of thiocyanate in order to stop forming of cyanide from sodium nitroprusside.<sup>13</sup> Measured by the amount of the inactivated cyanide, Sodium Thiosulfate is the most potent antidote. However, the effect is very slow, which is a problem in acute cases. In present study, Sodium Thiosulfate was given right from the beginning of the experiment as a bolus every six hours.

The drug was prepared by diluting 400 mg Sodium Thiosulfate in 100 ml NaCl.

Dosage: 5 x molar rate of SNP, i/v boli.

#### 3.2.5 Ilomedin<sup>®</sup>

Ilomedin (Iloprost Trometanolum, 50 µg/ml), Schering, Schweiz, AG is a synthetic prostacycline analogue, clinically used for treatment of thromboangiitis obliterans in humans. It is supposed that Ilomedin<sup>®</sup> will prevent platelet aggregation, adhesion and release reaction in a circumstance of lowered plasma arginine.

Preparation: 2.5 ml (50 µg) diluted in 47.5 ml saline.

Dosage: 50 mcg/day, via Orchestra Module DPS

#### 3.2.6 Aminosteril KE Nephro<sup>®</sup>

Aminosteril KE Nephro, Fresenius Kabi, CH – Stans 637, is used in the present study as an amino acid donor. It contains all essential amino acids except of arginine.

Dosage: 1 g/kg BW/day, via Orchestra Module MVP PT, infusion pump.

#### 3.2.7 Insulin

Insulin, 100 IE/ml (Novo Nordisk, A/S, DK – 2880, Denmark), is used in the present study for inhibition of the lysosomal pathway of protein breakdown.

Preparation: 2 ml (200 IE) diluted with 48 ml saline.

Dosage: 6 – 12 U/kg/day, via Orchestra Module DPS.

### 3.2.8 Glucose, 50% (Kantonsapotheke Zürich)

Given insulin will immediately sink the blood glucose level and lead to severe disturbance of conscience unless a sugar source is provided.

Dosage: 14 g/kg/day at beginning, then according to glycemia, via Orchestra MVP PT.

### 3.2.9 Normosang®

Normosang, ORPHAN EUROPE, 25 mg/ml, is used in the present study as a source of Heme, a protease inhibitor (see appendix IV).

Preparation: 6 mg/kg BW/day solved in 100 ml NaCl.

Dosage: ½ of the dosage in the first hour of the treatment and the second ½ in 12 hours. I/v administration, via a syringe.

### 3.2.10 Proferin ES

Proferin ES, Heme Iron Polypeptide, Colorado Biolabs, Inc., Nebraska  
Also used as a source of Heme.

Dosage: 3tbl in the evening before the start of the experiment (6 p.m.) and 3tbl two hours before anesthesia.

### 3.2.11 Metacam

Metacam, Meloxicam, 5 mg/ml, Boeringer Ingelheim Schweiz GmbH, 4002, Basel, is a Non – steroid anti-inflammatory drug, used in the present experiment to lower protein breakdown stimulated by a latent inflammatory process.

Dosage: 0.2 mg/kg/day

### 3.2.12 Dantrolene®

Dantrolene, P&G, Vifor Fribourg, is a direct – acting skeletal muscle relaxant, which probably interferes with the release of Ca++ from the sarcoplasmatic reticulum.  
In the present study the Dantrolene was used to inhibit protein breakdown.

Dosage: a quick i/v infusion, 2.5 mg/kg BW. Can be repeated until total 10 mg/kg BW

### 3.2.13 Sodium Lactate

Sodium Lactate, 4 Molar, Laboratorium Dr. G. Bichsel AG, Interlaken was given in the present study in a combination with lactic acid as a source of lactate.

Dosage: 25 ml/h at the beginning and then adjusted according to the plasma lactate level, via Orchestra Module MVP PT

### 3.2.14 Lactic Acid

Lactate acid, 4 Molar, Laboratorium Dr. G. Bichsel AG, Interlaken was given in the present study in a combination with sodium lactate as a source of lactate.

Dosage: 25 ml/h at the beginning and then adjusted according to the plasma lactate level, via Orchestra Module MVP PT

### 3.2.15 Antibiotics

Vancomycin (vancocin 250 mg, Eli Lilly) in a combination with Gentamicin was used in the present study to reduce the number of bacteria.

Dosage: vancomycin 5 mg/kg/day and gentamicin 2 mg/kg/day are given for gut sterilization. The antibiotics were given after bowel irrigation and two days before the dog was put in anesthesia.

Kefzol, Eli Lilly, cefazolin natrium equ. a 1 g, was used in the present study as a prophylactic antibiotic drug.

Dosage: 1 g/day, divided in four dosages, i/v.

### 3.2.16 Isosteril

Isosteril, Fresenius Kabi, Stans CH – 6371, was used as a electrolyte source.

Dosage: 1.5 – 3 ml/kg BW/h

### 3.2.17 Ringer Lactate

Ringer lactate solution, Fresenius Kabi, Stans CH – 6371 is used in the present study to maintain normovolemia.

Dosage: 1.5 – 3 ml/kg BW/h

### 3.2.18 Potassium chloride (KCL)

Potassium chloride (Potassium chloride 15%, Kantonsapotheke Zürich) was used to compensate the potassium that goes into the cells if a high dosage of glucose is administrated.

Dosage: 20 mmol/1 l of Isosteril

### 3.2.19 Morphasol

Morphasol (Butorphanolum 4 mg/10ml, D.E.Gräub AG, Bern), is used in the present study for sedation

Dosage: 0.007 mg/kg BW, i/m

### 3.2.20 Dormicum

Midasolamum 15 mg/3 ml (Roche Pharma AG, 4153 Reinach), also used for sedation in the present study

Dosage: 0.1 mg/kg BW, i/m

### 3.2.21 Propofol

Propofol 10 mg/ml (Fresenius Kabi), is used in the present study for induction of general anesthesia

Dosage: 2-4 mg/kg BW, i/v initially, 0.4 mg/kg BW/ minute for the maintenance of the anesthesia.

### 3.2.22 Isofluran 100%

Isofluran 100% (Forene, ABBOT AG, 6340 Baar), is used in the present study as an inhalation anesthetic.

Dosage: 1.2 minimal alveolar concentrations

### 3.2.23 Sintenyl

Sintenyl (Fentanyl, 50µg/ml, SINTETICA S.A., CH - 6850 Mendrisio), is used in the present study to regulate respiration of the dogs during the 24 hours of total anesthesia

Dosage: 0.045 – 0.3 µg / kg BW / minute

### 3.2.24 Vetanarcol

Vetanarcol (Pentobarbitalum natricum, 162 mg/ml, Veterinaria AG, Zürich), was used in the present study for euthanasia of the dogs, after the 24 hours of total anesthesia.

Dosage: 81 – 162 mg/kg, i/v

## 3.3 TECHNICAL EQUIPMENT

### 3.3.1 Infusion system machine

For continuous, simultaneous administration of several drugs in the present study, an infusion system machine named Orchestra<sup>®14</sup>, manufactured by Fresenius Vial S.A. Le Grand Chemin F – 3859 Brezins, France and delivered by Fresenius Kabi AG, CH – 6371 Stans, was used.

This infusion system is designed and used for the treatments in the intense care stations and anesthesia rooms. It is a modular system that gathers from 1 to 8 infusion devices. There are three functionally different modules of the system:

#### 1) The Base

The base is a data concentrator and power supply for up to 8 DPS and/or MVP models. It enables connection of the Orchestra<sup>®</sup> infusion workstation to a patient Data Management System via a single RS232 cable. Power supply from 95 to 240 V and 50/60 Hz. Battery life: 2 hours minimum. Communication interface compatible with major PDMS software.

The base connects all other modules in the infusion system, controls the whole system and stores the data about the patient and about all drugs/fluids administrated with the system. These functions of the base enable a proper work of all connected pumps and it records this work. One can read in the base at any time the infused quantities of any drug as well as the total balance of the infused fluids.



Orchestra<sup>®</sup> Base Primea

Figure 3: Orchestra Base and two DPS modules connected on the base

## 2) The DPS module

The DPS (Dynamic Pressure System) Model of the Orchestra infusion system is a precise syringe drive, which is connectable to the base. The flow rate selection ranges from 0.1 to 1200 ml/h, with a flow accuracy from  $\pm 1\%$  on drive mechanism and  $\pm 2\%$  on syringes. The pump has also a programmable bolus rate from 50 up to 1200 ml/h. If there is any objection in the infusion, an occlusion alarm and/or a disconnection alarm signal where the problem is.

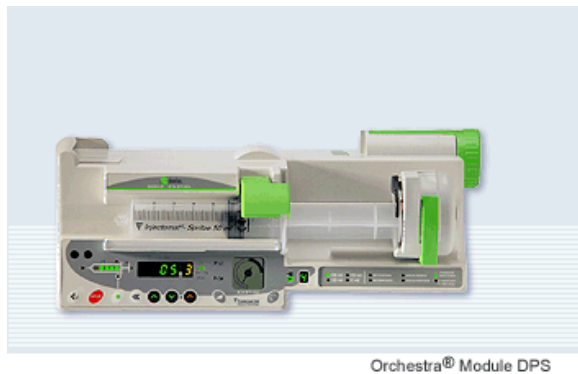


Figure 4: Orchestra® DPS Module

## 3) The MVP PT module

The Module MVP PT is a volumetric infusion pump that can be integrated into all Orchestra® configurations. Its flow rate ranges from 1 to 1000 ml/h and 0.1 to 100 ml/h in micro-infusion mode. The flow rate accuracy is  $\pm 5\%$  with recommended sets. The pump possesses different modes: micro or macro-infusion, ramp up/ramp down, sequential, induction, bolus and primary/secondary. The alarms for detection of pressure variations in the line allowing both faster occlusion detection and a line disconnection assure a safe work with this Orchestra® module.



Figure 5: Orchestra® MVP PT Module

### 3.3.2 Monitoring equipment

As dogs were in total anesthesia and were given drugs that could have compromised the vital function of the dogs, a wide range of vital signs were measured and documented: pulse rate, O<sub>2</sub> and CO<sub>2</sub> blood concentration, arterial and venous blood pressure both invasive and non – invasive, EKG, concentration of anesthetic gases in the lung alveoli and the body temperature. All these parameters were continuously monitored and documented by the anesthetic monitor AS3, designed and manufactured by Datex – Ohmeda, FIN – 00031, Finland (now GE Healthcare).<sup>15</sup>

### 3.4 BLOOD WORK

The blood of the experimental dogs was drawn from catheters for two reasons:

- 1) The amino acids in blood plasma were continuously monitored, especially arginine, ornithine, citrulline and 3 – methylhistidine. For this purpose 2 ml EDTA blood every two hours were taken from central venous catheter.
- 2) The most important, in blood measurable, parameters that might indicate side-effects of the given therapeutic protocol were also measured from the dogs' blood.  
In all dogs glycemia were measured every two hours by Glucometer Elite (Bayer) and the appropriate test strips. In addition, ACT (Activated Coagulation Time) was measured every two hours from the 0.4 ml arterial blood, means Hemochron R-401, a coagulation monitoring instrument, manufactured by Fresenius Kabi. Also, in dogs that were given a source of lactate, its plasma level was measured every two hours means Lactate Blood Test Meter and appropriate strips, Arcray, Inc., Japan. In this dogs it was also performed the blood gas analysis to determine the blood pH from 1 ml arterial blood in the heparinized syringe.

At the beginning of the experiments, at the beginning of the protocol administration, after 12 hours of the given protocol and just before end of the protocol, hematologic (hematocrit, hemoglobin, PCV, number and differentiation of leukocytes, MCV, MCHC, MCH and number of platelets) and biochemical (metabolites: total bilirubin, glycemia, urea, creatinine, proteins – biuret, albumin, cholesterol, enzyme: alkaline phosphatase, amylase, ASAT (GOT) and ALT (GPT), electrolytes: sodium, potassium, calcium, phosphor ) analysis from each experimental dog's blood were performed.

At the beginning and at the end of protocol administration the coagulation times (Quick, PTT and TZ) were determined. At the end of the experiment the ammonia in blood were determined.

For the hematology 2 ml EDTA blood was needed, for biochemistry – 3 ml of serum, for the coagulation tests – 5 ml citrate blood and for ammonia 2 ml EDTA on ice.

### 3.5 AMINO ACID ANALYSIS

#### 3.5.1 The Analyzer

The amino acid blood serum concentration was analyzed by the Biochrom 20 Amino Acid Analyzer, manufactured by Pharmacia LKB, Cambridge, England.

The amino acid analysis is based on continuous flow column chromatography where the sample was loaded onto a column containing a cation-exchange resin.

The Biochrom 20 Amino Acid Analyzer was controlled by a personal computer (PC 486 DX 2), using specially designed software (Biochrom 20 Version 1.2, Pharmacia LKB, Biochrom Ltd.). Data was recorded and analyzed by EZChrom – TM – Chromatography Data System, Version 6.4, Scientific Software, Inc., San Ramon, California, USA.

#### 3.5.2 Preparation of the samples for the analysis

0.5 – 1 ml of fresh blood was sampled into an EDTA coated tube in order to prevent clotting of the blood and then immediately put on ice, which stopped the arginase activity in the blood sample. After at least 10 minutes, the samples were centrifuged for a minute in a table top centrifuge (3000 g), to separate plasma from the cellular components. Afterwards, 400 µl of the supernatant plasma were transferred to an Eppendorf tube and mixed with 200 µl of 10% 5 – sulfosalicyl acid for the protein precipitation. The Eppendorf was incubated then for 30 minutes at 4°C. Then, the mixture was centrifuged for 10 minutes. The supernatant was removed with a syringe and filtrated through Millex – GV4 – r, 0.22 µm, Millipore Products Division, Bedford, USA into a new Eppendorf.

So prepared samples were immediately analyzed or stored in a refrigerator.

For analyzing in the Biochrom analyzer, the sample was loaded in the specially designed capsules for the analyzer. A capsule was rinsed before sample loading with a loading buffer, Pharmacia Biotech, Ltd., Cambridge, England. Afterwards, 40 µl of the sample was pipetted into the capsule. Finally, 10 µl of the loading buffer was added into the capsule.

The capsule was added then into the autoloader of the analyzer and the amino acid measurement commenced automatically.



### 3.6 EXPERIMENTAL PROCEDURE

All planned experiments were conducted sequentially: there was only one dog in the experiment at a time.

#### 3.6.1 Preparation Phase

##### 3.6.1.1 Accommodation

Dogs that were objects of the present study accommodated in the facility named Stieghof, 8425 Oberembrach, in which all experimental dogs used by the Veterinary School Zurich are accommodated.

Each dog was brought into the Veterinary School Hospital Zurich on the day before the experiment started. The dog was fed (Pedigree Pal, dry food) and given Vancomycin, p/os in order to eliminate gut bacteria as a source of arginine in the evening before the experiment. In the next morning, dog was clysterized.

##### 3.6.1.2 Anesthesia and Catheterization

One hour before the experiment begin, the dog was clinically examined (pulse, respiration, heart and lung auscultation, mucosa inspection) and according these and, previously done, laboratory findings decided whether the dog can be put in total anesthesia. If the findings were favorable, the dog was sedated (Dormicum/Morfazol), inducted (Propofol) and maintained in anesthesia (Isofluran/O<sub>2</sub>/Air) for 24 hours.

In the jugular vein was put MultiCath, double – lumen central vein catheter, VYGON B.P.7 – 95440 ECOUEN, France, length 20 cm, flow rate 55 and 70 ml/minute.

The line for the central vein pressure measure was connected to the proximal flow of the central vein catheter.

In the cephalic vein was put Surflo i/v. catheter, 20 G, 1 ¼", Terumo.

In the tarsal artery was put arterial catheter, Arterial Canula with FlowSwitch<sup>®</sup>, 20 G, Becton Dickinson, UK. The line for the arterial pressure was connected to this catheter.

In the bladder was put a urine catheter, Arnolds<sup>®</sup>, 2.0 mm OD, 50 cm, SIMS Portex Ltd CT21 6JL, UK. This catheter was connected with a urine bag with a reflux valve, manufactured by MEDINORM AG, Germany.

The dog body temperature was measured by a probe, which was a integral part of the Datex – Ohmeda monitoring system. The probe was put in the dog's mouth and continuously measured the body temperature. If the hypothermia occurred, a water blanket, on which dog was put during anesthesia, was heated.

### 3.6.2 Drugs infusion

The drugs for infusion were prepared as planned. The infusion lines of all pumps were connected to the central one. The Orchestra<sup>®</sup> infusion system was started and pumped for a while to mix all drugs in the line before connecting to the central vein catheter in the dog's jugular vein.

At least one veterinarian monitored the dog, anesthesia and the infusion system for the whole experiment time.

The veterinarian did also the all needed blood work for the analyses.

### 3.6.3 Euthanasia and Pathology

As these were terminal experiments, after 24 hours of the drug protocol application, all dogs were euthanized with Vetanarcol, without waking the dogs from anesthesia.

The dogs were then autopsied (pathology, standard) and histopathology (liver, kidney, gut, lungs, and heart)) at the Institute for pathology, Vetsuisse Faculty University of Zürich under supervision of Prof. Dr. A. Pospischil.

The results of the post-mortem examinations were very similar to the results of pathologic examinations performed by Prof. Dr. J. A. Laissue on mice treated with a similar protocol (arginase, insulin, glucose). (Please see Caroline Sonja Walder, Inaugural – Dissertation).

Macroscopic findings were abnormal only in a few dogs, which died from bleeding. In these dogs hematorax, hemoperitoneum, blood in lungs, stomach and guts were observed. However, no signs of DIC were noticed.

In all other dogs no pathological findings, which could correlate with infused protocol, were observed. In one dog an old diaphragmatic rupture was found. In some others lung emphysema and/or pulmonary edema were found.

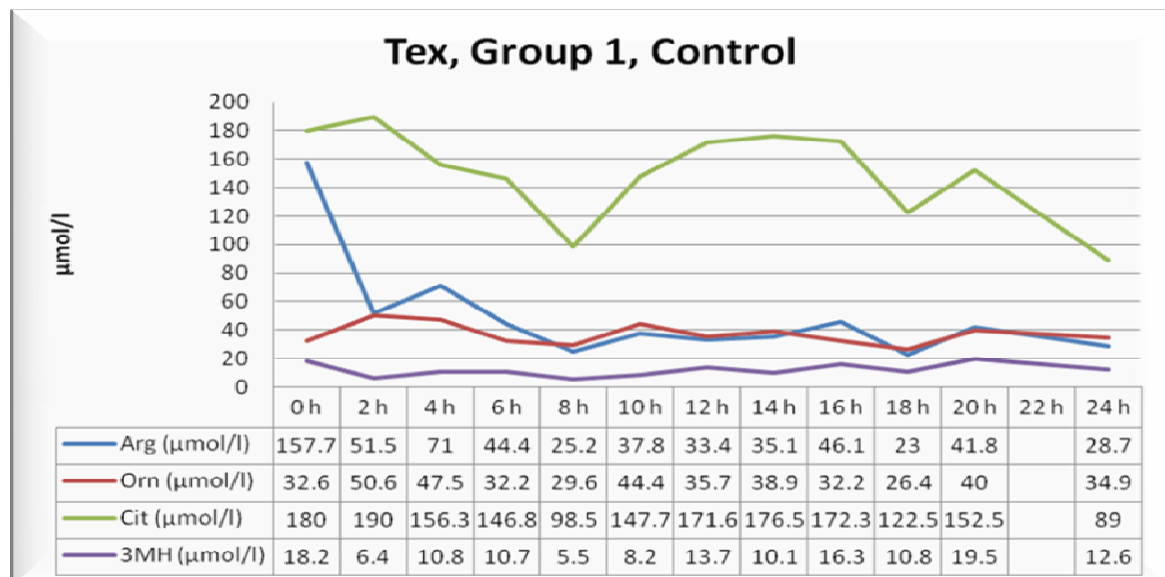
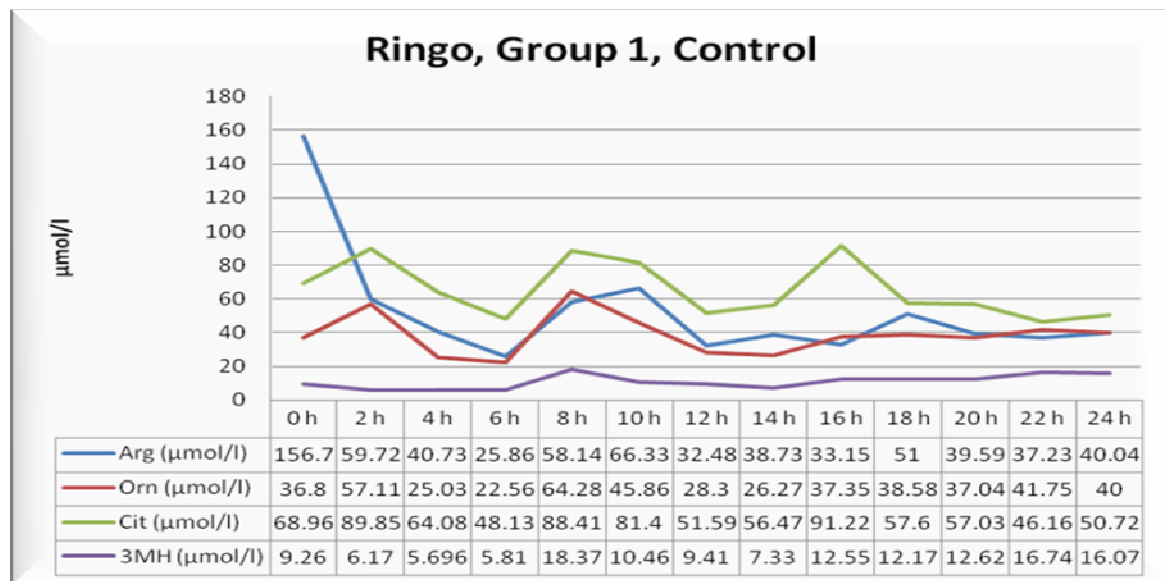
Histopathologic examination has also proved the findings of Prof. Laissue on mice. The most dogs treated with our anti-cancer protocol developed a moderate to marked changes in hepatocytes in form of a high intracellular glycogen concentration – defined by Prof. Laissue as fatty changes in hepatocytes and by Prof. Pospischil and his team as hepatitis.

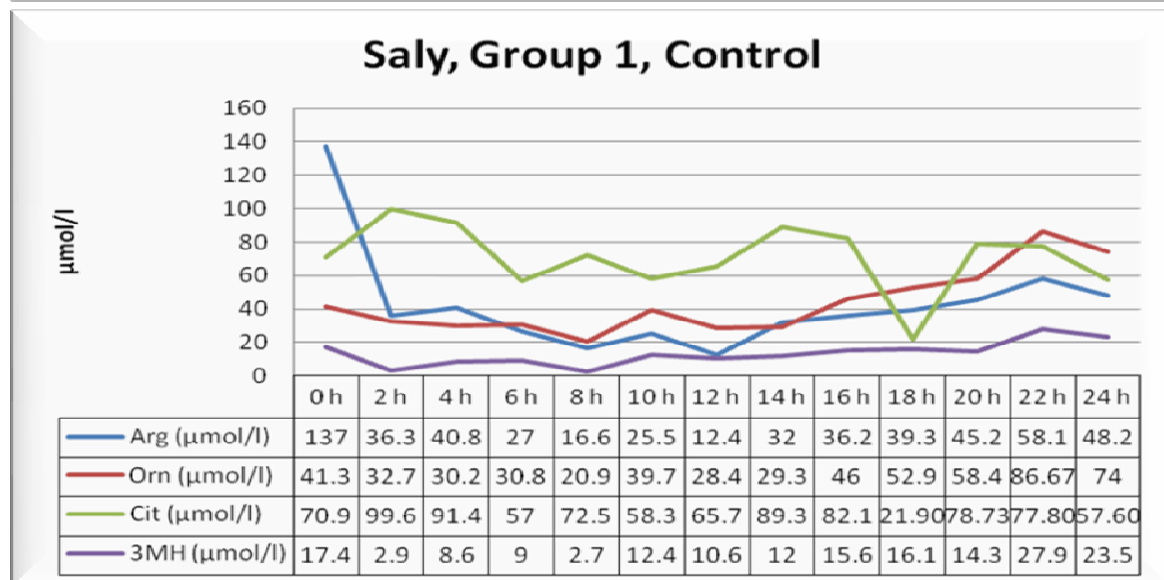
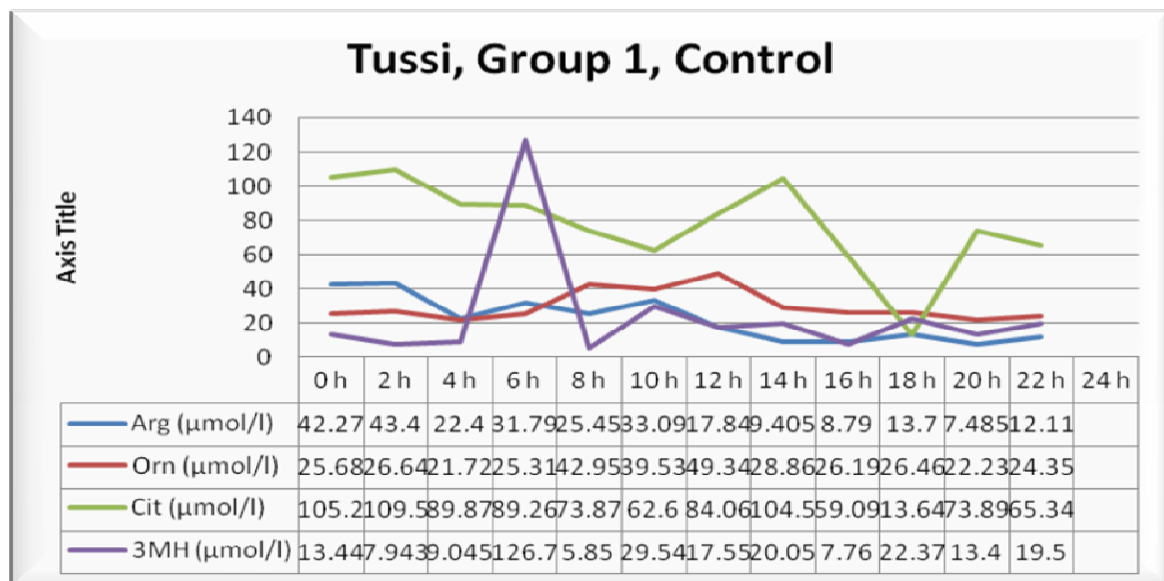
## 4 RESULTS

### 4.1 Group 1 – The Control group –

In the group 1 ("the control group"), four dogs were treated with arginase and the protocol that prevents side effects of arginine depletion. (SNP, Vasopressin, Thiosulfate).

The arginine, ornithine, citrulline and 3 – methyl histidine plasma levels of the dogs in the group 1 (Ringo, Tex, Tussi, Saly) are presented in the following graphs.

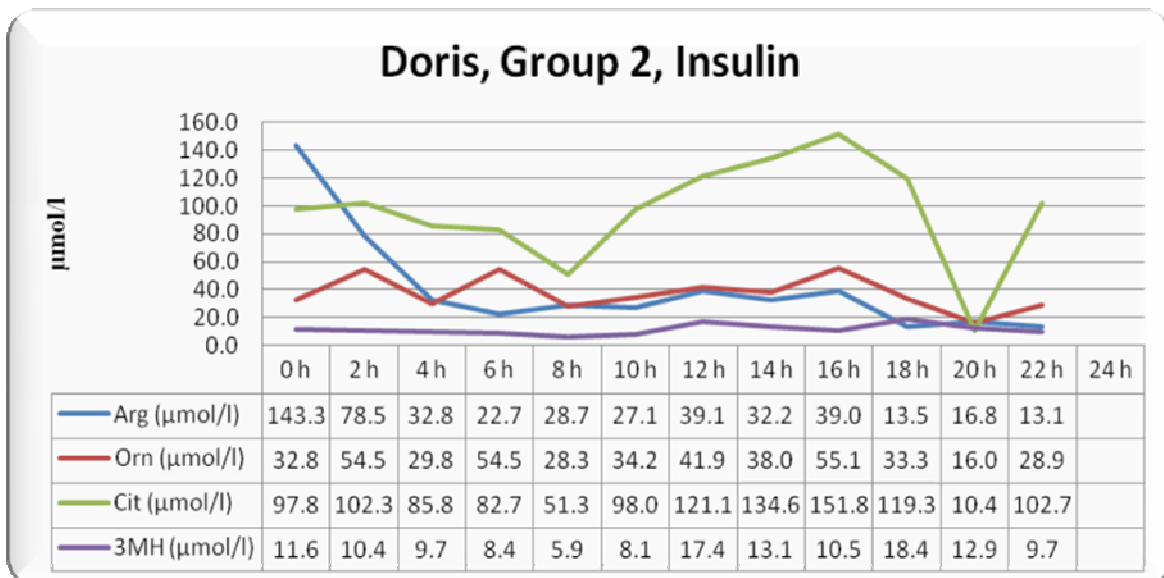
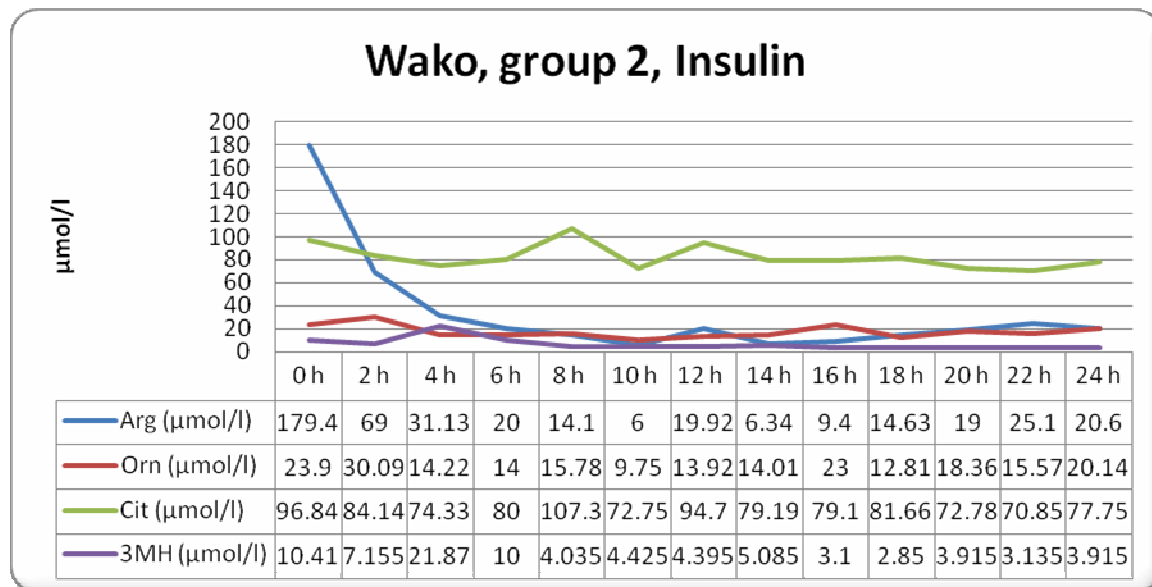




The average plasma level of arginine in the last 12 hours of all four experiments was around 40, the ornithine level was on average 35, the level of citrulline was around 65, and this of 3 methyl histidine approximately 15  $\mu\text{mol/l}$ .

## 4.2 Group 2 – The Insulin group –

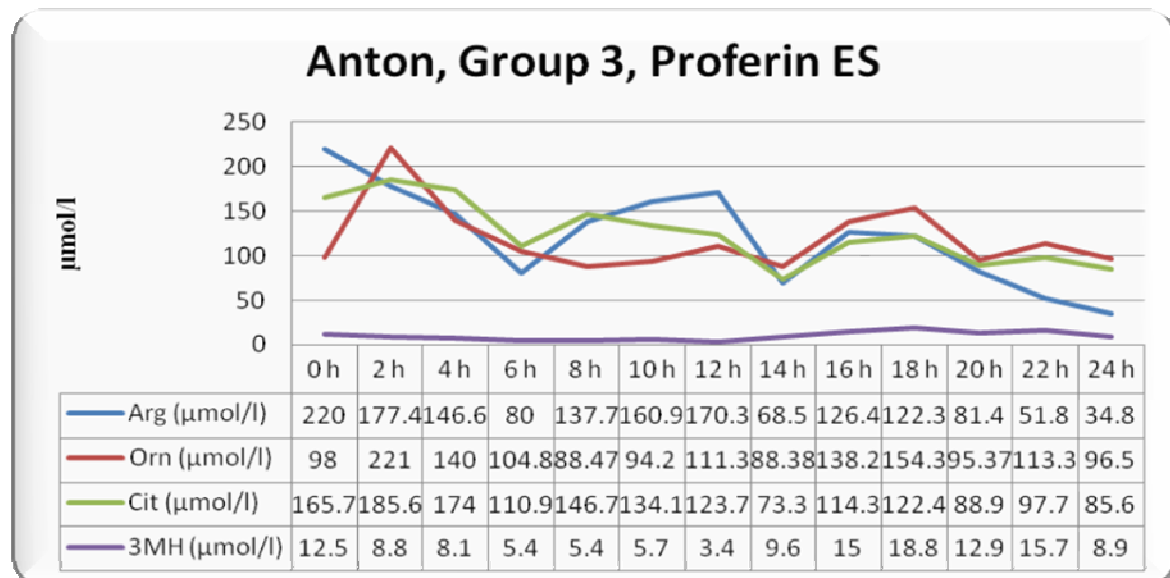
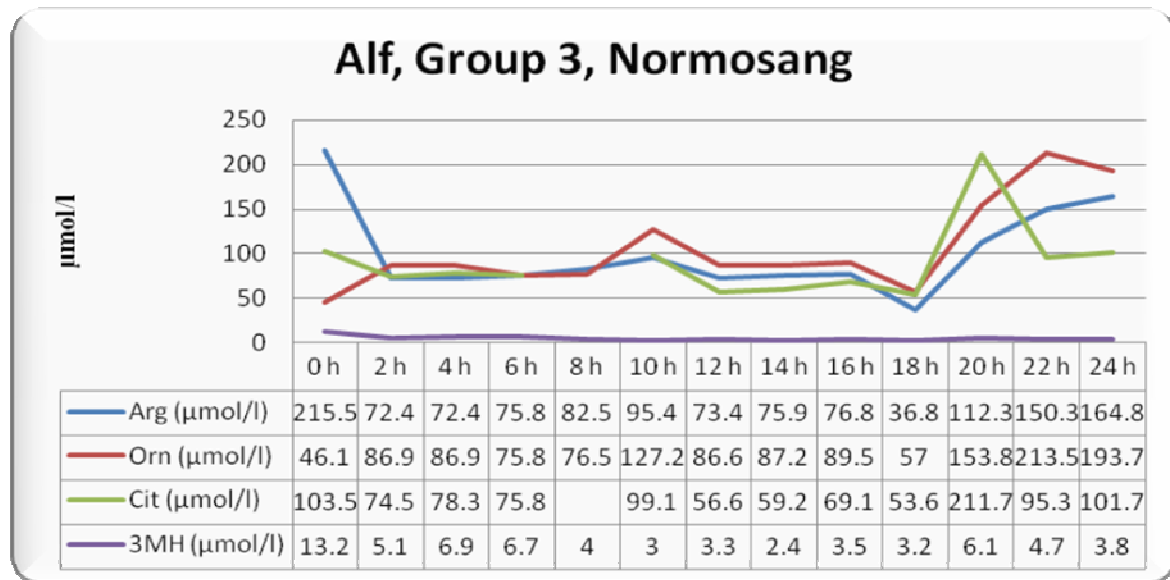
In the group 2 ("The Insulin group"), two dogs (Wako, Doris) were treated with the same protocol as the "control group" was, plus "insulin group" did receive insulin as well.



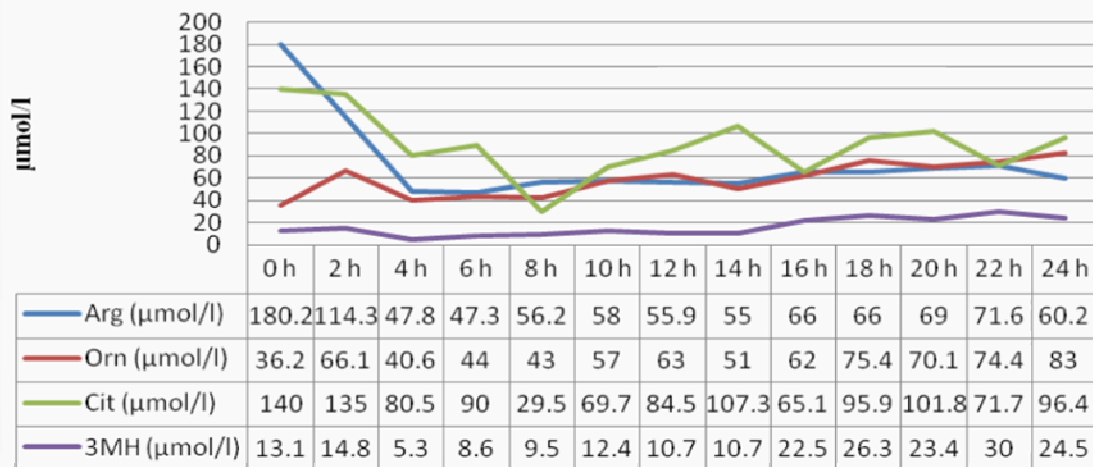
The average plasma level of arginine in the last 12 hours of both dogs was around 25, the ornithine level was on average 30, the level of citrulline was around 80, and this of 3 methyl histidine approximately 10 μmol/l.

### 4.3 Group 3 – The Proteasomal inhibitor group –

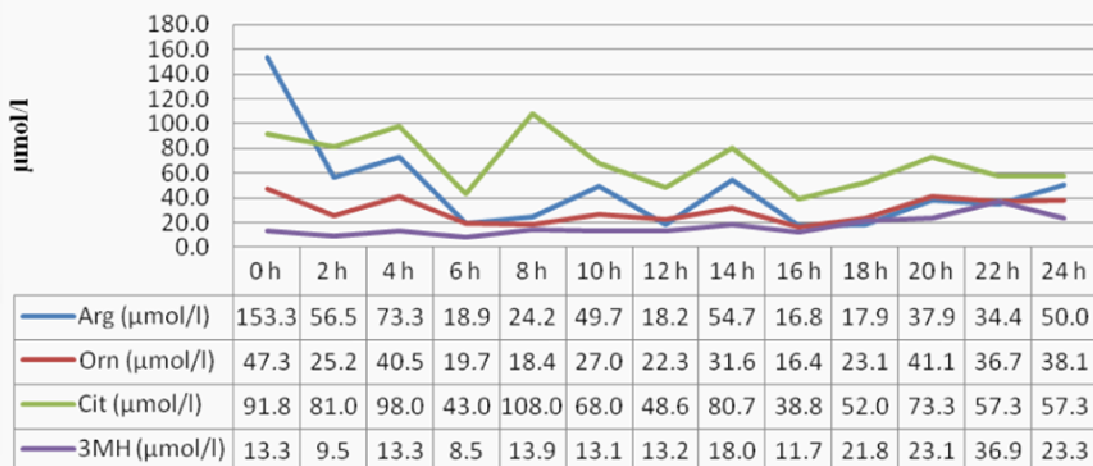
In the group 3 (– The Heme group –), four dogs (Alf, Anton, Pluto and Leo)) were treated with the same protocol as the – Control group – was, plus –The Heme group – did receive a Heme source – either Normosang or Proferin ES:



### Pluto, Group 3, Proferin ES



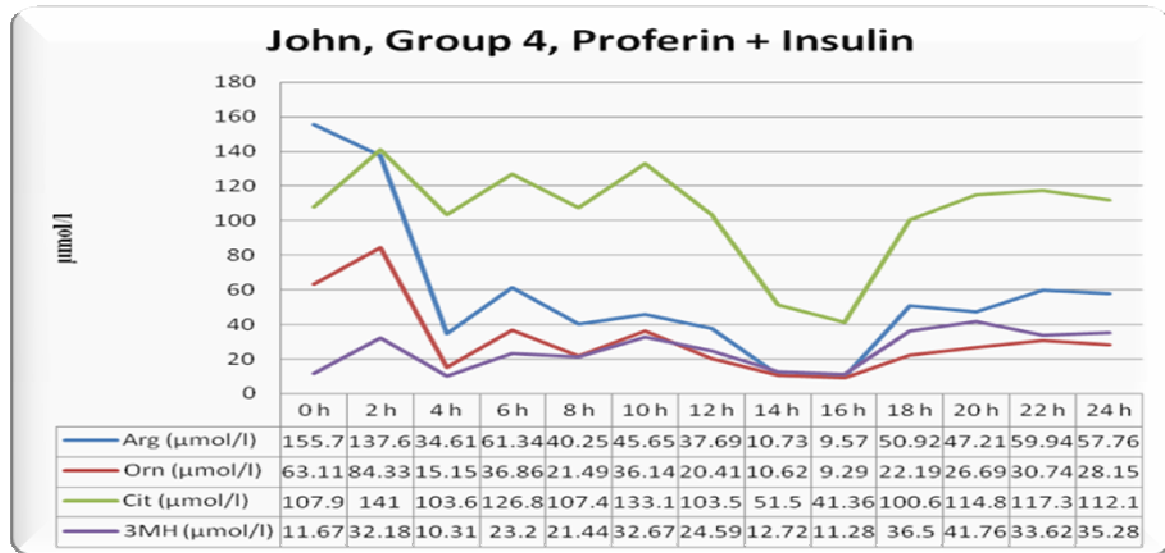
### Leo, Group 3, Proferin ES



The average plasma level of arginine in the last 12 hours of the all four dogs was around 70, the ornithine level was on average 70, the level of citrulline was around 90, and this of 3 methyl histidine approximately 15 µmol/l.

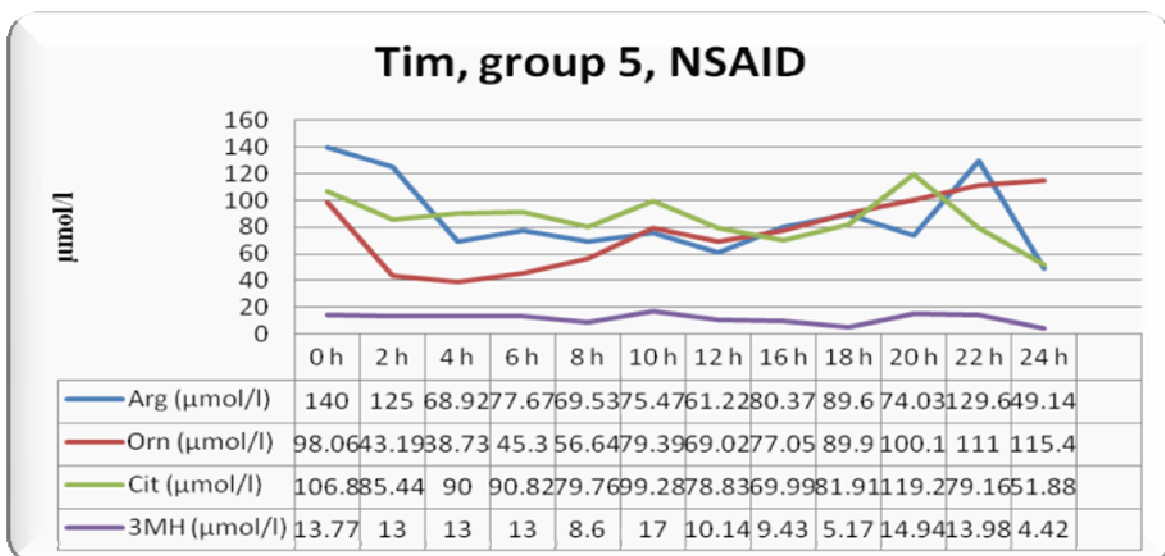
#### 4.4 Group 4 – The Insulin + proteasomal inhibitor group –

In the group 4 (– The Insulin + Heme group –), one dog (John) was treated with the same protocol as – The Control group – was, plus it did receive insulin and a Heme source – Proferin ES. As we did not see any improvement of urea cycle amino acid plasma levels depletion, we did not treat additional dogs with this protocol combination.



#### 4.5 Group 5 – The NSAID group –

In the group 5 (– The NSAID –), one dog (Tim) was treated with the same protocol as the “control group” was, plus it did receive a NSAID - metacam. As we did not see any improvement of urea cycle amino acid plasma levels depletion, we did not treat additional dogs with this protocol combination.

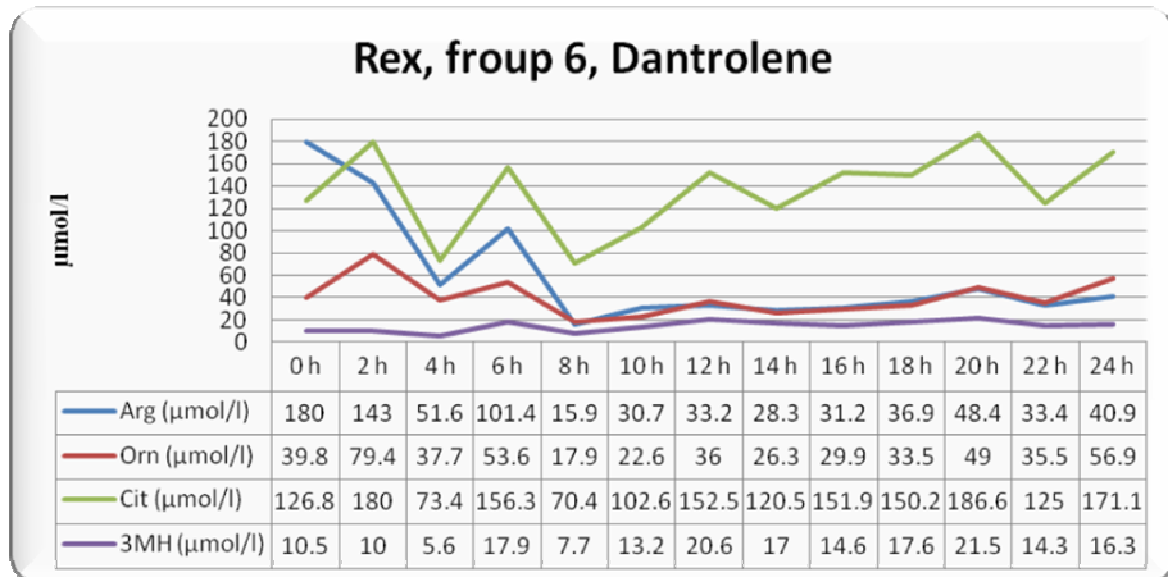




#### 4.6 Group 6 – The Dantrolene group –

In the group 6 (– The Dantrolene group –), one dog (Rex) was treated with the same protocol as the “control group” was, plus it did receive Dantrolene.

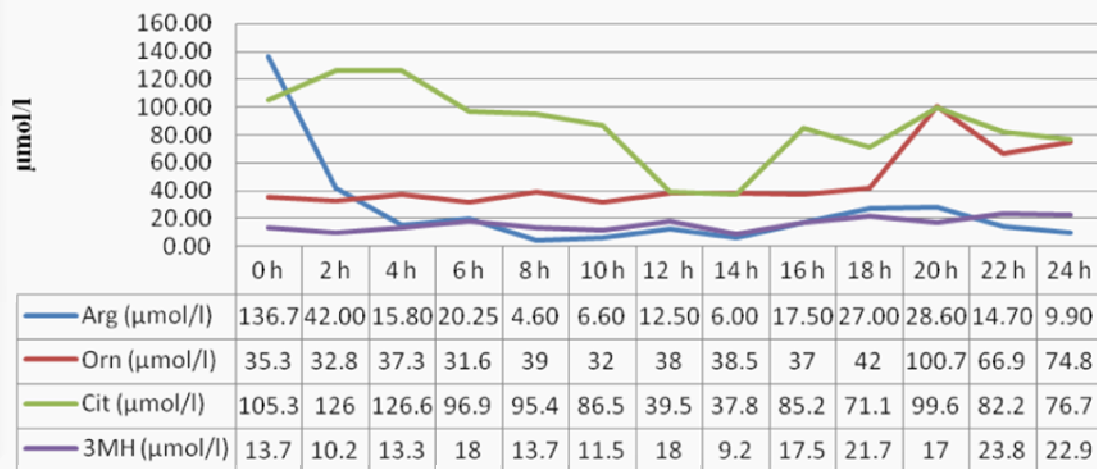
As we did not see any improvement of urea cycle amino acid plasma levels depletion, we did not treat additional dogs with this protocol combination.



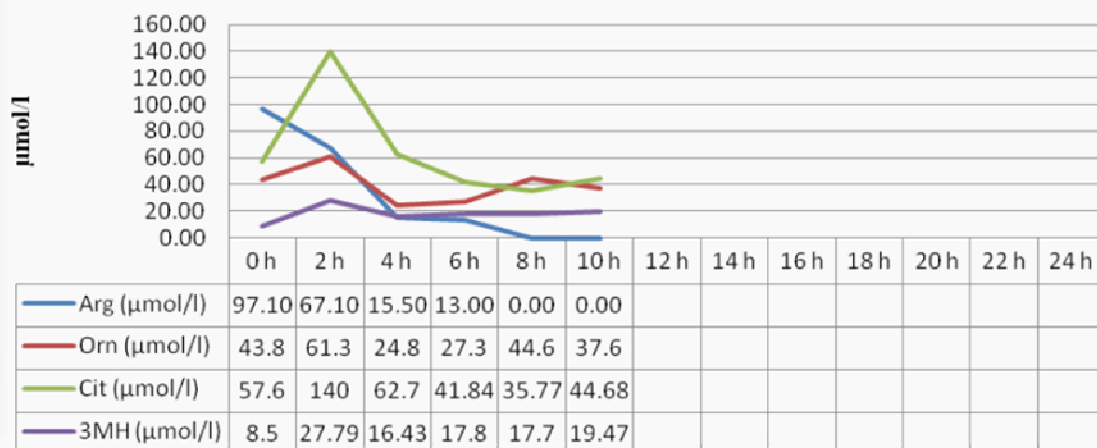
#### 4.7 Group 7 – The Lactate group –

In the group 7 (–The Lactate group –), four dogs (Ina, Sam, Ina, Arni, Kim, Tina, Bob, Maya and Ken) were treated with the same protocol as – The Control group” was, plus they did receive a lactate source – either Na – lactate or DI Milk acid (DL MA).

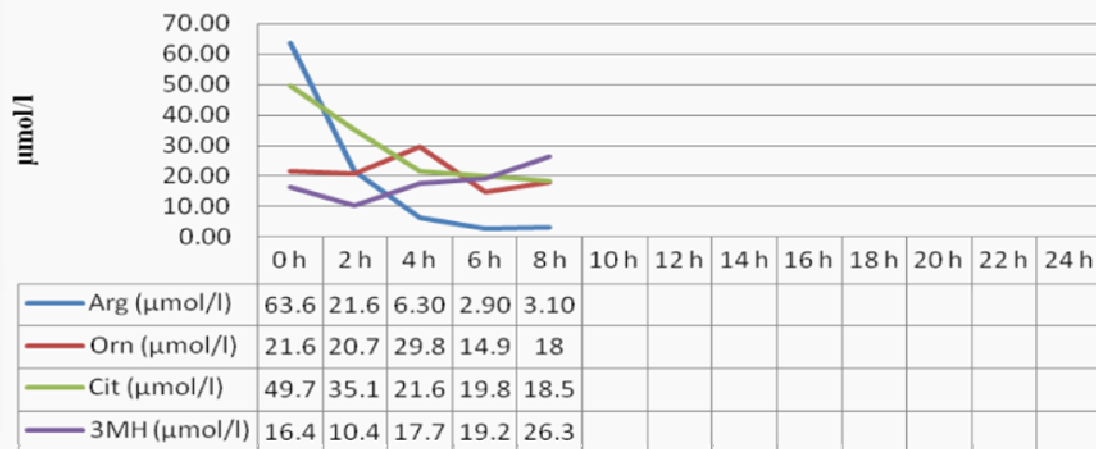
### Ina, group 7, Na - lactate



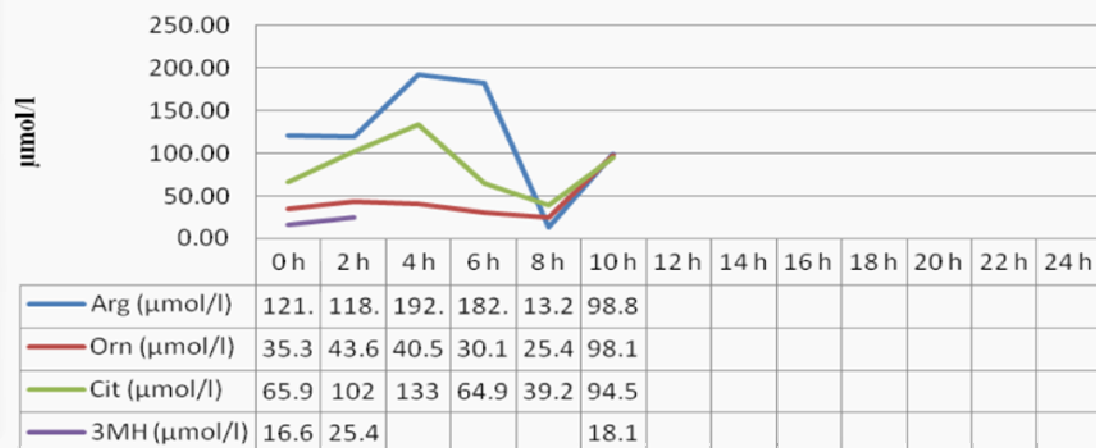
### Sam, group 7, Na - lactate



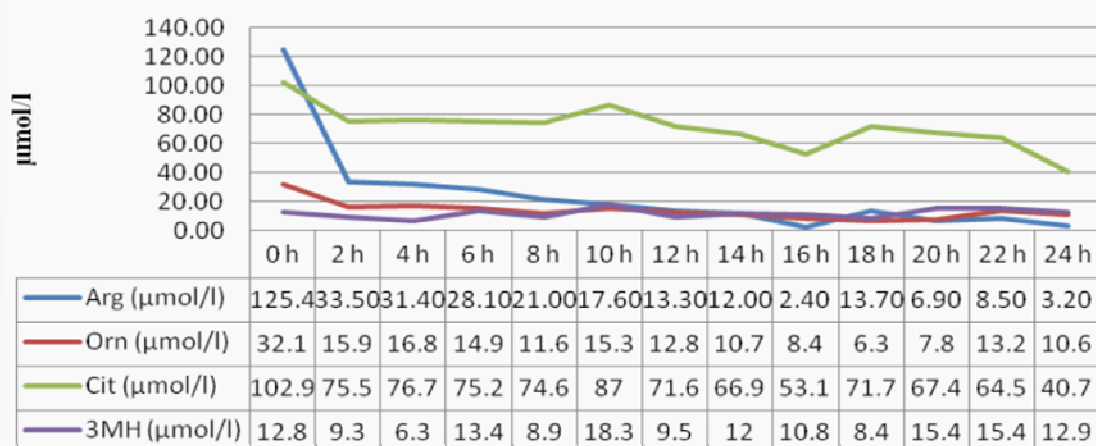
### Arni, group 7, Na - lactate, Insulin



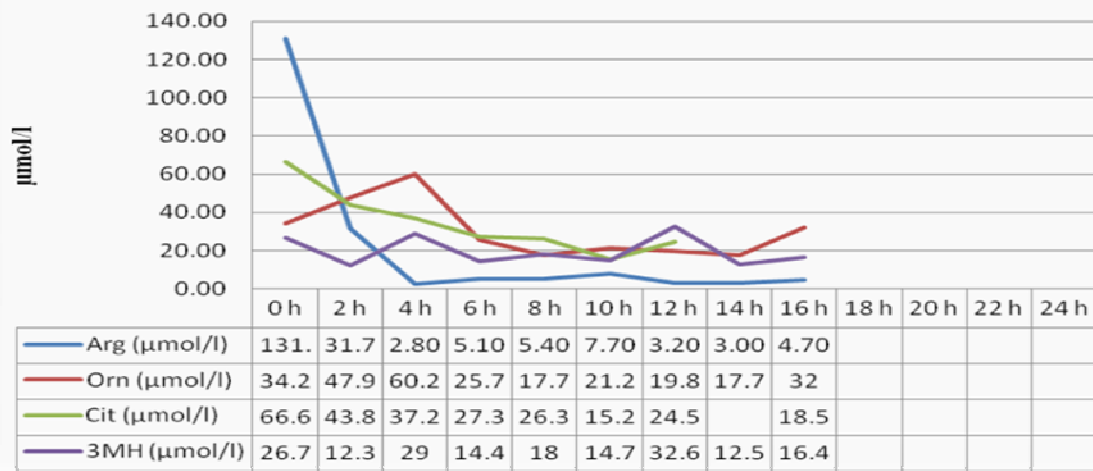
### Kim, group 7, Na - lactate, Insulin, HCl



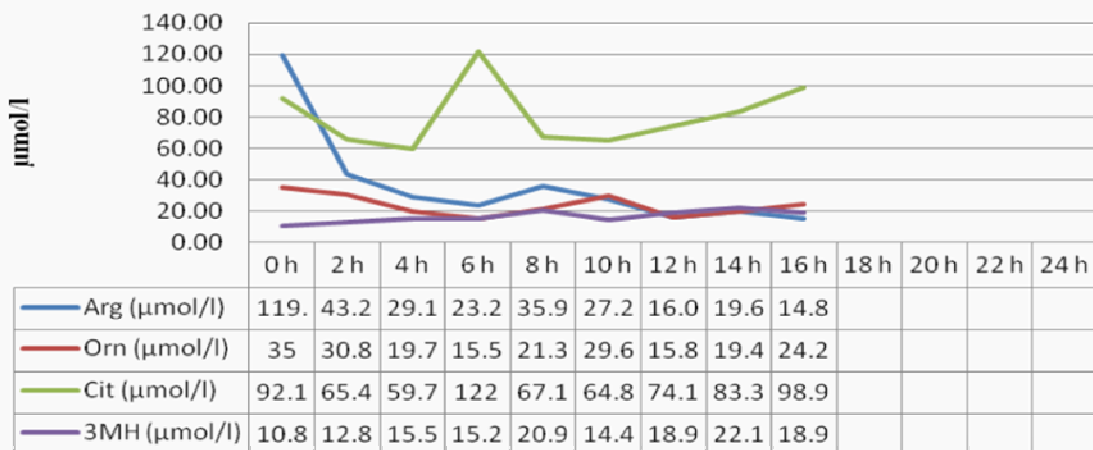
### Maya, group 7, Na-lact., Insulin, DL MA

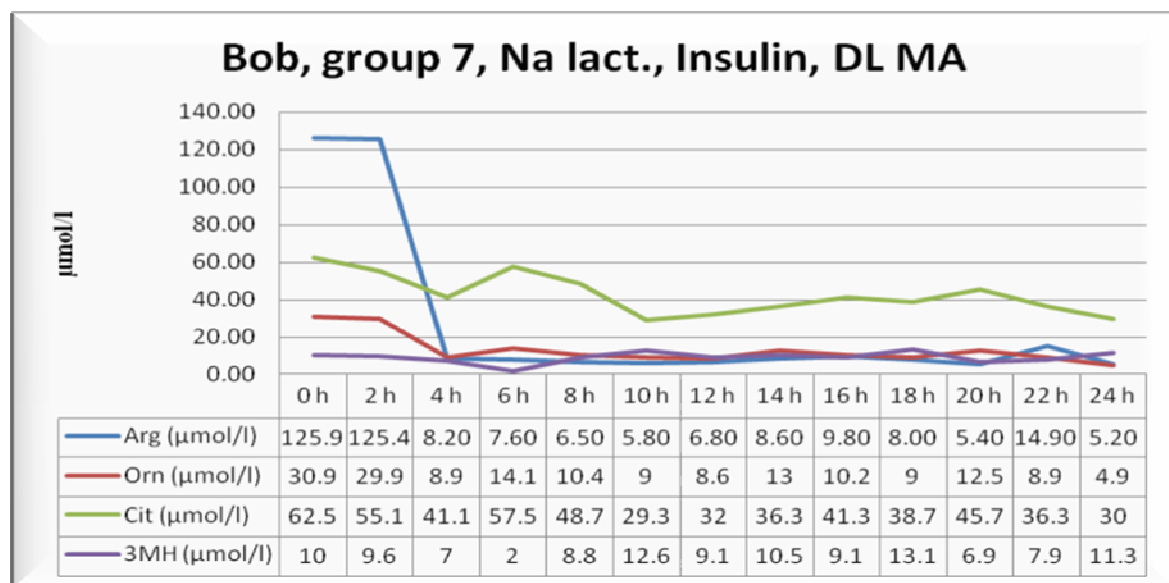


### Ken, group 7, Na-lact., Insulin, DL MA



### Tina, group 7, Na-lact., Insulin, DL MA

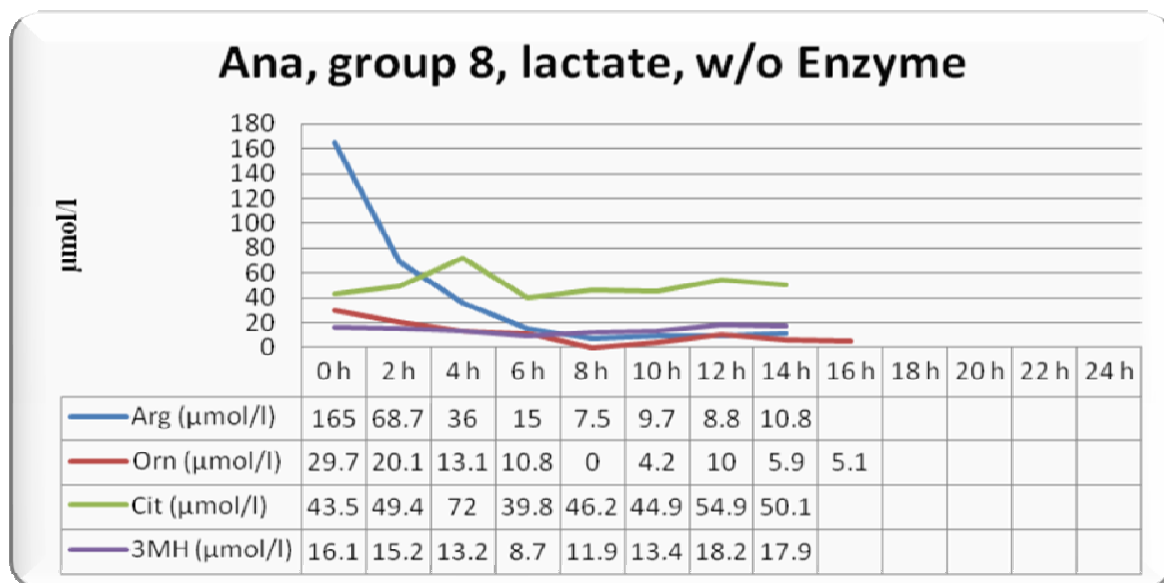
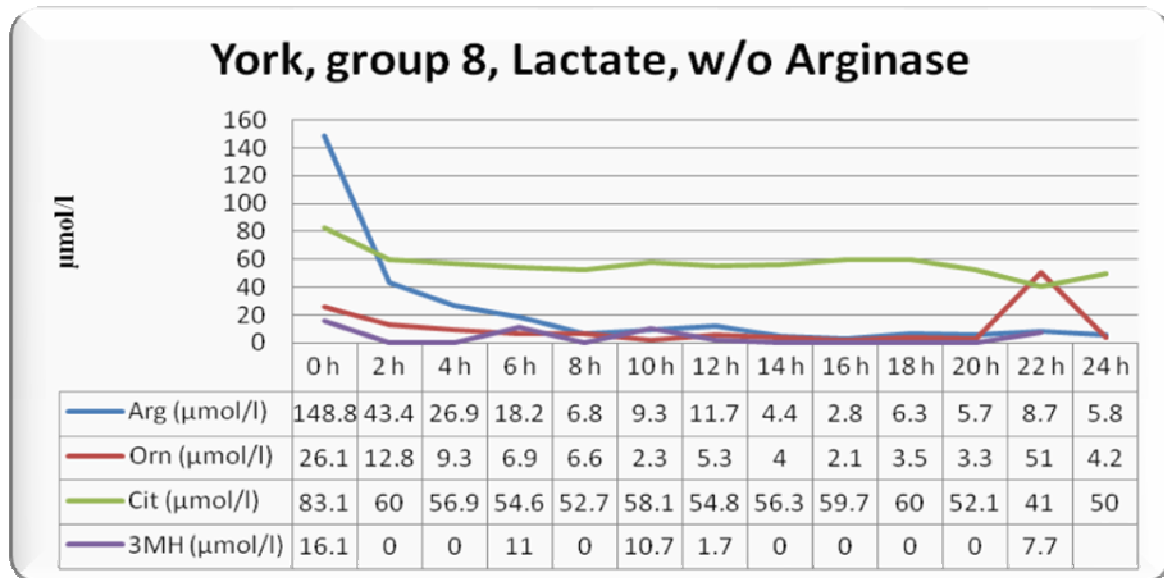




The average plasma level of arginine in – The Lactate group – in the last 12 hours of all eight dogs was around 8.75, the ornithine level was on average 10, the level of citrulline was around 50, and this of 3 methyl histidine approximately 10 μmol/l.

#### 4.8 Group 8 – No Arginase group –

In the group 8 (– No Arginase group –), two dogs (York and Ana), were treated with the same protocol as – The Control group – was, but without arginase. They also received lactate sources – Na – lactate and DI Milk acid (DL MA).



The average plasma level of arginine in the – No Arginase group – in the last 12 hours of all eight dogs was around 8, the ornithine level was on average 10, the level of citrulline was around 50, and this of 3 methyl histidine approximately 10 µmol/l.

## 5 DISCUSSION

This series of challenging experiments had the goal to examine whether arginine, ornithine and citrulline plasma levels are completely depletable *in vivo*, in dogs, which had never been conducted before. The starting hypothesis that arginase, coupled with protein breakdown inhibition, -no arginine, ornithine and citrulline diet- and the inhibition of *de novo* arginine synthesis, will lead to a total depletion of arginine and the other urea cycle intermediaries, was formed according to the previous experiments done partially on mice and dogs by the same group at Vetsuisse Faculty, Zurich and according to the relevant scientific literature.

The experimental procedure has proved to be complex but manageable if carefully planned and executed. The experimental dogs, anesthesia, medicaments, equipment and veterinarians have to be well prepared in advance to minimize errors that can be fatal to the tested animals as well as for the objectivity of the obtained results.

It was crucial that all applied medicaments either for achieving urea cycle amino acids depletion or for the amelioration of the side effects were prepared as described and administrated in the proposed dosages.

In addition, a 24-hour-permanent monitoring of the vital functions of the experimental dogs is an imperative that were able to assure a minimum of adverse effects.

It was also important that the procedure of amino acids measurements was done properly in order to convey the right result.

Generally speaking, this series of experiments has proved some results of previously conducted experiments at the Vetsuisse Faculty, Zurich, and published articles about the *de novo* arginine synthesis in mammals. However, the main hypothesis, that a total (below the level of detection) depletion of circulating arginine, ornithine and citrulline by tested protocols was not proved positively.

Nevertheless, these experiments did neither prove that the depletion of urea cycle amino acids by controlled diet, a complete inhibition of protein breakdown and a complete inhibition of *de novo* synthesis of ornithine, citrulline and arginine is impossible.

This study revealed some evidences that such a complete depletion is indeed possible if all factors that contribute to the arginine plasma homeostasis are anticipated and inhibited.

Let us discuss results of every experimental group.

### 5.1 Results of –The Control group–

This aim of this experimental group, which did not obtain any of the inhibitors of the endogenous sources of arginine, was to serve as a reference for the other groups that did receive such inhibitors. The dogs in “the control group” only received arginase and drugs for amelioration of the side effects caused by low circulating arginine.

The results of this experimental group proved to be as expected: arginine plasma level was around 40  $\mu\text{mol/l}$ , this of ornithine 35, citrulline 65 and 3 – methylhistidine (3MH) 15.

It is expected that arginase given i/v, cannot significantly deplete circulating arginine if the homeostatic mechanism such as protein breakdown and *de novo* arginine synthesis are not inhibited.

The experiments also proved that the drugs administrated to prevent arginine depletion side effects are well tolerated by dogs in the applied dosages.

## 5.2 Results of –The Insulin group–

This group has obtained insulin as a protein breakdown inhibitor in addition to the protocol that received “the control group”.

It was expected that insulin will lower the plasma level of arginine and ornithine but not citrulline (not contained in muscles). The results indeed proved the expectations: arginine plasma level decreased to 25  $\mu\text{mol/l}$ , ornithine remained around 30, citrulline raised to 80 and the 3MH decreased to 10.

As 3MH level raises if protein breakdown is in place, its decreased level indicates that insulin inhibited protein breakdown.

The results of this group also proved that insulin has no influence on the circulating citrulline level, as citrulline is not contained in muscles and so its plasma level does not depend on the protein breakdown.

## 5.3 Results of –The proteasomal inhibitors group–

The aim of this experimental group was to give evidences whether proteasomal inhibitors will lower circulating levels of urea cycle amino acids, as it known that a good amount of proteins is degraded in this proteolytic pathway.

However, the average results of circulating urea cycle amino acids were higher than in –The insulin group– (arginine 70, ornithine 70, citrulline 90, 3MH 15).

The plasma level of 3MH in dog that received Normosang was the lowest of all experiments. However, 3MH is predominately found in urine and no treatment has shown a significant decrease of 3MH in urine.

## 5.4 Results of –The insulin plus proteasomal inhibitors group–

As insulin is described as an inhibitor that predominantly affects the lysosomal proteolytic pathway, it was intriguing to see if any synergy of combined inhibitory effects of insulin and proteasomal inhibitors can influence the plasma level of urea cycle amino acids.

However, the results (average arginine 50, ornithine 30, citrulline 100 and 3MH 20) did not proved expected synergy.

The experiment was designed properly to exclude a possible mistake of insufficient resorption of the proteasomal inhibitors: two different inhibitors were used – Normosang for i/v administration and Proferin ES for p/os administration.

The reason of the lack of synergy could rather be that insulin also inhibits the proteasomal proteolytic pathway effectively (see Appendix IV).



### 5.5 Results of – The NSAD group –

It is well known that in infection and notably sepsis there is an increased protein breakdown of skeletal muscle protein and reduced rate of protein synthesis.<sup>16</sup>

As NSAID have in higher doses, besides their analgesic and antipyretic, also anti – inflammatory action. The aim of this experimental group was to prove whether there is an effect of a NSAID (Metacam) on protein breakdown.

The results revealed that Metacam does not influence the plasma levels of arginine, ornithine, citrulline or 3MH in a healthy dog without signs of infection / inflammation.

### 5.6 Results of – The Dantrolene group –

The intracellular proteins can be degraded in skeletal muscle in the absence of ATP and if lysosome function is prevented by weak bases. In this case,  $\text{Ca}^{2+}$  and calpains play a major role in the enhanced proteolysis.<sup>17</sup>

Dantrolene, an anti malignant hyperthermia drug, which decreases release of calcium from sarcoplasmic reticulum, is known as an inhibitor of  $\text{Ca}^{2+}$ - dependent proteolytic pathway. In particular in sepsis, Dantrolene does greatly decreases protein breakdown<sup>18</sup>.

The aim of testing Dantrolene was to examine the role of the  $\text{Ca}^{2+}$ - dependent proteolytic pathway in arginine homeostasis.

The results of the plasma level amino acids of the one experimental dog did not show that Dantrolene makes any difference in a healthy dog's urea cycle amino acid plasma levels.

### 5.7 Results of – The Lactate group –

Lactate is known as inhibitor of proline oxydase, an enzyme that catalyzes the first step of ornithine production from proline in the enterocytes (see Appendix V, table 7).

This production of ornithine, which is converted into citrulline in enterocytes, and citrulline into arginine in the kidney is well known as 'intestinal – renal' arginine biosynthesis axis.<sup>19</sup>

Inhibition of the 'intestinal – renal' axis seems to be inevitable if the goal is arginine urea cycle amino acids plasma level below detection.

Exactly such a condition (arginine and citrulline plasma concentrations below detection limit) have been reported in the infant (2 – year – old girl) with elevated plasma concentration of lactate (hyperlactacidemia; up to 14 mmol) due to an inherited deficiency of pyruvate dehydrogenase activity.<sup>20</sup>

The aim of these experimental group was to imitate similar condition by increasing lactate plasma level in the experimental dogs to achieve inhibition of the 'intestinal-renal axis' and deplete arginine, ornithine and citrulline.

The results indeed proved that lactate (in particular in combination with insulin – see the results of dogs Maya, Ken, Bob, Tina) can be a strong mediator of arginine and ornithine plasma levels.

Unfortunately, lactate did not have such an effect on citrulline plasma level as it had in the infant with hyperlactacidemia (citrulline below detection!).

The question is why did the citrulline plasma level remained stable? Was kidney function of conversion of citrulline to arginine still in place or it was blocked due to e.g. anesthesia and low kidney perfusion? Or do dogs have different and more stable homeostatic mechanisms for maintaining citrulline plasma level relatively high compared to this in humans?

The question is also what the role of glutamine in ornithine production is? According to Wu et al (see appendix V, figure 9), glutamine is another source (beside proline) for ornithine production. Is it possible that an inhibition of this pathway would lead to a complete depletion of citrulline? Is it possible that such an inhibition occurred in the infant?

## 5.8 Results of – The Lactate group –

A question arised after the group 7: What proportion of the arginine depletion is due to arginase and what due to inhibition of the endogenous sources of arginine, namely protein breakdown and 'intestinal – renal axis'?

Two dogs were treated with the same protocol as the group 7, but without enzyme arginase.

Surprisingly, the results of the plasma level of arginine in this group were not higher than the arginine plasma level that did receive arginase!

The question is if the activity testing of arginase was really accurate? Was the amino acids measurement procedure accurate, or the amino acid analyzer always measured some amino acids even in the empty capsules?

## 6 CONCLUSIONS

In spite of some opened scientific and technical questions, the conclusions drawn from this series of experiments are:

1. It is possible to deplete circulating arginine and ornithine below 10  $\mu\text{mol/l}$ , even without an active, enzymatic arginine depletion.
2. Side effects, seen in dogs as consequences of low level of arginine, can be ameliorated by the therapeutic protocol developed by Dr. Tepic et. al.
3. Circulating citrulline cannot be significantly depleted by any of tested protocols.
4. Circulating arginine, ornithine and citrulline cannot be depleted below 1  $\mu\text{mol/l}$  by any of in this study tested protocols, without further modifications.

## 7 APPENDIX I: CANCER

### 2.1 Human Cancer

#### 2.1.1 Definition of cancer

Cancer (medical term: malignant neoplasm) is an abnormal growth of cells which tend to proliferate in an uncontrolled way, to invade (intrude on and destruct of adjacent tissues) and, in some cases, to metastasize (spread to other locations in the body via lymph or blood). These characteristics differentiate a malignant tumor - cancer from a benign tumor, which is self-limited, does not invade nor metastasize.<sup>21</sup>

Cancer is not a single but a group of more than 200 different and distinctive diseases. Cancer can involve any tissue of the body and have many different forms in each body area.

Beside infectious diseases, most illnesses have multifactorial etiology. Cancer is no exception. In other words, there is no single cause for any one type of cancer. Scientists talk about cofactors: carcinogens like tobacco, genetic makeup, genetic mistakes, poor immunity, viruses, diet and environmental factors all or some of them like pieces of a puzzle come together and promote disease of cancer<sup>22</sup>. All cancers begin in cells or, more precisely, are consequence of the changed cells' growth and division ability.

#### 2.1.2 Types of cancer

Cancer types can be grouped into broader categories. The main categories of cancer include:

Carcinoma - cancer that begins in the skin or in tissues that line or cover internal organs.

Sarcoma - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

Leukemia - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

Lymphoma and myeloma - cancers that begin in the cells of the immune system.

Central nervous system cancers - cancers that begin in the tissues of the brain and spinal cord.

Germ cell tumor – tumors derived from totipotent cells.

Blastoma – tumors which resemble immature of embryonic tissue.

### 2.1.3 Cancer epizootiology

A 2008 report from the USA leading cancer organizations<sup>23</sup> shows cancer death rates decreased on average 2.1 percent per year from 2002 through 2004, nearly twice the annual decrease of 1.1 percent per year from 1993 through 2002.

Estimated new cases and deaths from cancer in the United States in 2008:

New cases: 1,437,180 (does not include non-melanoma skin cancers)

Deaths: 565,650

Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007. Cancer is responsible for about 25% of all deaths in the U.S. and is a major public health problem in many parts of the world.

In the U.S., lung cancer causes about 30% of cancer deaths but only about 15% of new cancer cases; the most commonly occurring cancer in men is prostate cancer (about 25% of new cases) and in women is breast cancer (also about 25%).<sup>24</sup>

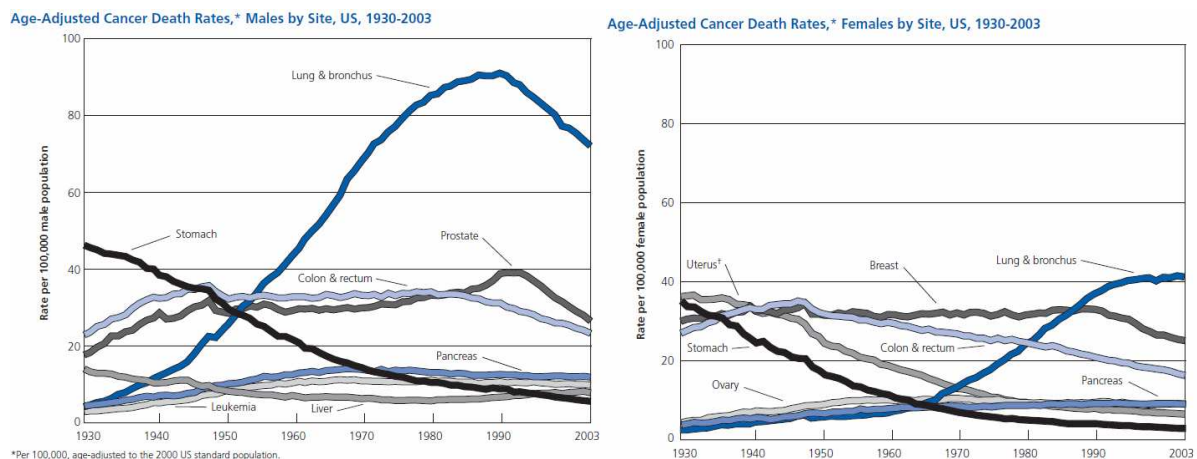


Figure 3: The leading death cause cancers in males (left) are lung, prostate and colon and rectum cancers. The leading death causes cancers in females are lung and breast cancer.

Cancer can also occur in young children and adolescents, but it is rare (about 150 cases per million in the U.S.), with leukemia being the most common.<sup>25</sup>

### 2.1.4 Cancer diagnosis

There are many signs and symptoms that may indicate the presence of cancer. These may be observed directly, through imaging technologies or confirmed by lab tests. However, these signs and symptoms of cancer may resemble those of other conditions and only pathohistological findings of a biopsy can establish, or rule out, a diagnosis of cancer.

### 2.1.5 Cancer treatment

As there are more than 200 types of cancer, and there is not a sufficient treatment for any type of cancer that cannot be removed surgically, there is variety of treatment protocols for different types and stages of cancer as well as general state of the patient. Some of these protocols are established as 'gold standard', i.e. treatments that currently achieve better results in terms of years of survival and life quality than the other treatments, but still not curing the disease.<sup>26</sup> Beside the surgery, which is the most effective treatment for cancer, oncologists try to prolong patients' life as long as possible with chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. A number of experimental cancer treatments are also under development.

Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy and radiation is often limited by toxicity to other tissues in the body. Unfortunately, the success of cancer treatment is still measured by the Kaplan – Meier survival curve, which shows what proportion of patients survive 1, 2, 3, 4 and 5 years.

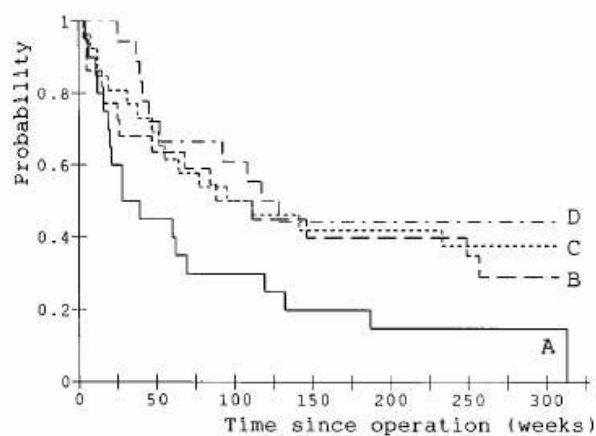


Fig. 2 Survival curves of patients with squamous cell lung carcinomas (Kaplan-Meier estimates), subdivided according to different groups A–D (see legend to Fig. 1).  $P = 0.04$ , by log-rank analysis.

Figure 4: A typical graph from a scientific journal<sup>27</sup>, reporting a cancer treatment results

### 2.1.6 Cancer prevention

Over a third of cancer deaths worldwide are due to potentially modifiable risk factors, which are headed by tobacco smoking, alcohol use and diets low in fruit and vegetables.

In developed countries overweight and obesity is also a leading cause of cancer and in low-and-middle-income countries sexual transmission of human papillomavirus is a leading risk factor for cervical cancer.<sup>28</sup>

Cancer prevention is defined as active measures to decrease the incidence of cancer.

It is usually defined as either primary prevention, for people who have not been diagnosed with a particular disease, or secondary prevention, aimed at reducing recurrence or complications of a previously diagnosed illness.

There are several groups of attempts to prevent cancer or to lower its prevalence. Some of them include avoiding carcinogens or altering their metabolism, pursuing a lifestyle or diet that modifies cancer-causing factors, medical intervention (chemoprevention, treatment of pre-malignant lesions), genetic testing, vaccination and/or screening.

## 2.2 Psychosocial impact of cancer

Although still not the killer # 1, cancer is, together with AIDS, an illness that produces all the negative emotions coupled with a deadly disease: fear, anger, pain, hurt, desperation and even guilt. Most cancer patients need great deal of support to cope with this devastating mood or adjustment disorders.

Cancer has a profound psychological impact on the quality of life (QOL) of patients and their families, on family and social relationships, and on role functioning. The challenging anticancer therapy, which is often coupled with severe side effects, consumes the intellectual and emotional energy of patients and their families.

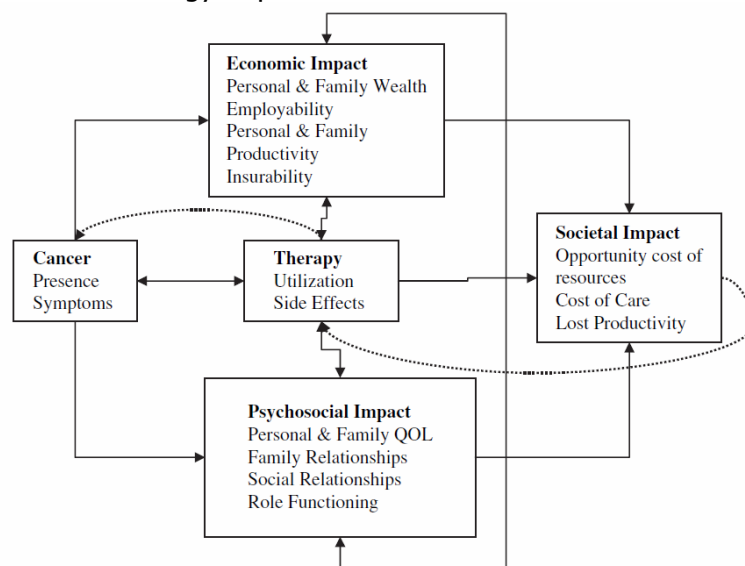


Figure 5: The interrelated impacts of cancer on patients and their families<sup>29</sup>

The QOL of cancer patients is a multidimensional construct including mental and emotional well-being, physical limitations, the availability of appropriate treatment, family and social relationships, and finances. The dimensions are intertwined, and each is important to cancer patients for maximal functioning. QOL is affected by the type and extent of cancer and by the chronic concerns of patients that their cancer will recur or progress.

A poor QOL means reduced productivity and employability of patients and their families.

Cancer poses an enormous financial burden on patients and their families, particularly on society's most economically and socially vulnerable groups such as low-income and uninsured families. In a national survey of elderly Americans, low-income patients undergoing cancer treatment spent approximately 27% of their annual income on out-of-pocket medical expenses.<sup>30</sup> In addition, a recent survey of households affected by cancer showed that nearly half who did not have consistent health insurance during cancer treatment reported using all or most of their savings to pay for cancer care. Twenty-seven percent had to delay or forgo cancer treatment because of the costs, and 6% filed for personal bankruptcy.

Cancer also affects the employability and productivity of patients and their families. The 19% of the households affected by cancer reported that the disease caused someone in the household to lose or change job or to work fewer hours. In addition, the results of a national survey to determine the effects of commonly occurring chronic conditions on work in the adult population demonstrated that cancer was associated with the highest reported rate of work impairment and the largest number of impaired work days of all conditions.<sup>31</sup>

A study by Yabroff and colleagues showed that the estimated cost for the cancer patient's time spent traveling, waiting for appointments, and receiving services or procedures during the first 12 months after diagnosis was as much as \$5605.<sup>32</sup>

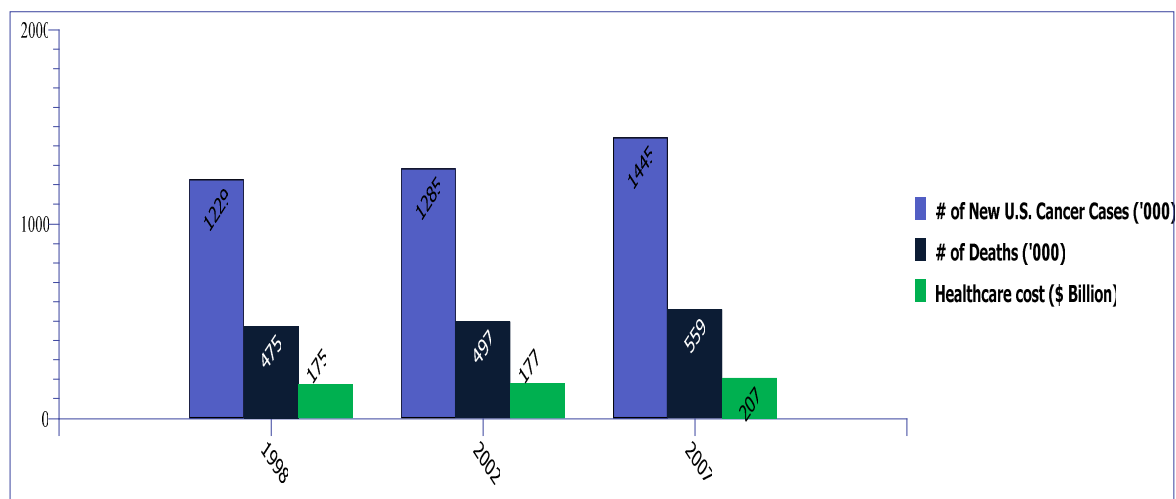


Figure 6: Human and Economic Burden of Cancer

## 2.3 Cancer in dogs

Cancer is a major cause of pet animal death. This is difficult to document but some studies supported this statement. In a series of 2000 presented necropsy cases, 23% of the patients died of cancer. Moreover, 45% of dogs that lived to 10 years or longer died of cancer.<sup>33</sup> The prevalence of cancer in pet animals is increasing. This frequency of cancer in pets is increasing for several reasons, but it is mostly related to animals living to older and older ages. Dogs have 35 times as many skin cancers as do humans, 4 times as many breast tumors, 8 times as many bone cancers, and twice as high an incidence of leukemia.<sup>34</sup>



The cancerous dogs have for the pharmaceutical industry a twofold importance. Firstly, there is a market called veterinary oncology. There are roughly one million new dog cancer cases diagnosed and managed each year in the U.S.A<sup>35</sup>, costing each pet owner between \$3000 and \$5000. Secondly, pet animals with spontaneously developing cancer provide an excellent opportunity to study many aspects of cancer from etiology to treatment. Cancerous dogs may unlock clues to improving the outlook for this disease in both animals and humans. There is a great deal of studies supporting this statement.

## 8 APPENDIX II: ARGININE

Arginine is a unique amino acid: only arginine is defined as a semi-essential amino acid in healthy adults. It is essential as it plays crucial roles in many vital biochemical processes and as its plasma level is constant, tightly regulated by a number of homeostatic mechanisms. However it is semi – essential as it can be synthesized from non essential, ubiquitous amino acids glutamine and proline.

This semi – essential character of arginine, implies its complex biochemistry both its catabolism and its anabolism, which is clearly described in the inaugural dissertation written by my colleagues Caroline Sonja Walder and Anja Irene Gödl.<sup>36</sup>

It is remarkable how versatile arginine in animal cells is, serving as a precursor for proteins, nitric oxide, urea, polyamines, proline, glutamate, creatine and agmatine.

On the arginine source site, the important roles play dietary intake, protein breakdown and de novo synthesis.

The arginine metabolism is complex and highly regulated set of pathways that are still incompletely understood both on the whole-body and cellular levels.

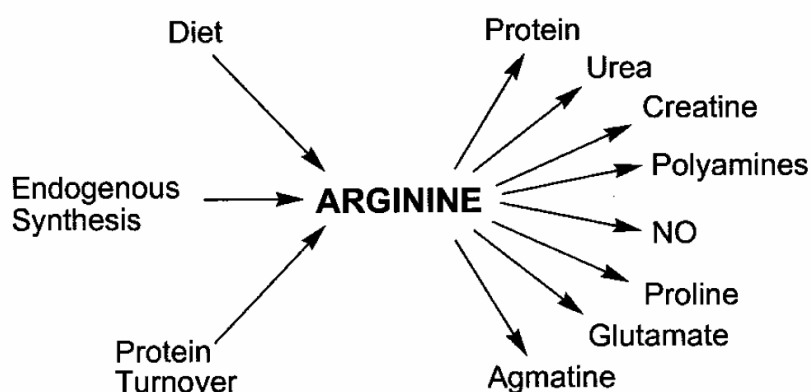


Figure 7: Arginine serves as precursor in synthesis of protein, NO and at least 6 additional metabolically important compounds in mammals.<sup>37</sup>

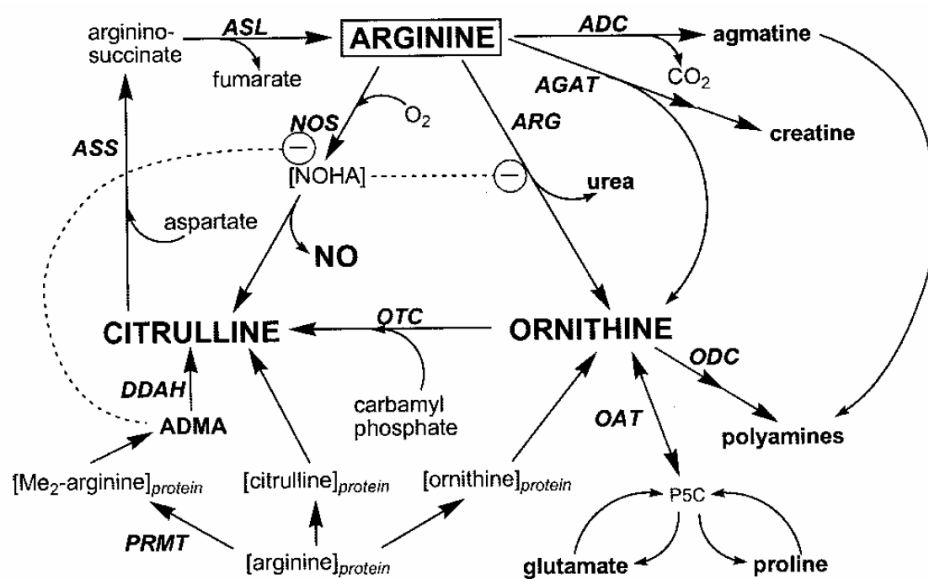


Figure 8: Arginine is produced by a single enzyme, but serves as a substrate to four

Arginine is not only a versatile precursor, but it is also metabolically interconvertible with the amino acids citrulline, ornithine, proline and glutamate!<sup>38</sup> The all these complex processes of arginine multiple metabolic fates are still not perfectly and completely pictured, but it is obvious that in order to deplete arginine level in the extracellular space, one has to consider the potential precursors of arginine and fight them too.

## 9 APPENDIX III: PROTEIN BREAKDOWN

As said before, the goal of the Tepic's cancer research group has been to kill cancer by restricting the availability of urea cycle amino acids (arginine, ornithine and citrulline) to cancer cells. This goal implies a total deprivation of the urea cycle amino acids in blood plasma and the extracellular liquid that surrounds the cancer.

However, this attempt will naturally face a powerful enemy in a life organism – homeostatic mechanisms that keep the plasma and extracellular level of amino acids in a constant range.

### 9.1 Amino Acid homeostasis

As with most nutrients, plasma amino acid concentrations in physiological situations vary within fixed limits and are tightly regulated. Interestingly enough, not only the plasma levels of essential amino acids but also these of non – essential amino acids are kept in a fixed range!

One of the reasons for such a strict control of the maximal plasma level of amino acids is the possible damaging effect of amino acid to the brain tissue. The brain is very sensitive to AA excess as several AA or their metabolites are mediators having inhibitory or stimulating neurological effect.

On the other hand, a minimum level of amino acids in plasma is also strictly maintained, assuring the amino acids availability as substrates for protein building and numerous metabolic reactions that involve amino acids.

Plasma AA concentrations at any particular time are the result of their rate of appearance (Ra) and their rate of disappearance (Rd).<sup>39</sup> The Ra is the sum of AA intake and AAs released by tissues, notably muscles. The Rd is the sum of AA metabolism and oxidation, incorporation of AAs in proteins and loss in urine, feces. Interestingly, the contribution of mentioned factors in the AA homeostasis is different due to different presence of AA in proteins and in the diet. Lysine plasma level, for example, mostly comes from protein degradation and diet.<sup>40</sup> In contrast, the contribution of de novo synthesis to Ra is much higher for AAs such as citrulline or alanine.<sup>41</sup> This is favorable for our anti – cancer approach, as interventions that deplete urea cycle amino acids do not dramatically influence levels of other amino acids, which would consequently have severe side effects.

Most of the plasma arginine comes from the muscle protein breakdown. In order to keep arginine, ornithine and citrulline plasma levels under 1  $\mu\text{mol/l}$ , it is essential to prevent protein breakdown.

### 9.2 Muscle protein breakdown

Skeletal muscle is the largest pool of protein in the body. This protein mass is maintained with a very dynamic and well balanced processes of protein synthesis and protein degradation.

It seems that protein breakdown is too important for a mammal organism to be constrained to only one proteolytic pathway. Thus, there are multiple pathways of protein degradation occurring on different proteolytic systems. These systems are strictly regulated to ensure that a

highly selective continual proteolysis. While most liver proteins turn over once a day or once in two days, some regulatory enzymes do not live longer than 15 minutes.

This continual, regulated protein destruction serves several important homeostatic functions. Firstly, destruction of numerous regulatory proteins enables a mammalian cell to adopt its structure to the physiological conditions. In addition, protein breakdown provides an essential quality control mechanism that selectively eliminates abnormally folded or damaged proteins.

It is estimated that an average human adult synthesizes and degrades approximately 1.0 – 1.5 kg protein per day.<sup>42</sup> Therefore, it is very challenging to completely control such a powerful system, by inhibiting its proteolytic side and stimulating its anabolic side.

### 9.2.1 Lysosomal proteolytic system

The most extensively studied proteolytic system in mammalian cells is the lysosomal pathway. Lysosomes are cell organelles, which contain a large number of acidic proteases (cathepsins B, H and D) and other acid hydrolases.

The proteolytic activity of the lysosomal apparatus is in particular visible under poor nutritional conditions, such as lack of amino acids or in absence of insulin. In such conditions enlarged lysosomes called “autophagic vacuoles” are forming, which uptake and hydrolyze many cytosolic proteins. According to Furuno and Goldberg as well as Lowell et al., the lysosomal pathway is mainly involved in degrading surface membrane proteins and endocytosed extracellular proteins. Lysosomal pathways play not an important role in cytosolic protein degradation. Insulin and IGF – 1 suppress autophagic vacuole formation.

### 9.2.2 $\text{Ca}^{2+}$ – dependent proteolytic system

At neutral pH, two  $\text{Ca}^{2+}$ – dependent proteases called calpains I and II, show appreciable cytosolic proteolytic activity, especially in the damaged tissues.

Although this nonlysosomal proteolytic system is very potent, its function under normal conditions or in atrophy is quite unclear.

### 9.2.3 Mitochondrial proteases

A complete system for protein turnover within the mitochondrial matrix, digest organellar proteins and polypeptides using ATP energy.

### 9.2.4 ATP – dependent proteolytic system

In most cells, there is an energy – dependent proteolytic system. When ATP in muscle is depleted by some inhibitors of oxidative phosphorylation, the breakdown of cell proteins falls by 60 – 70%. However, such an effect is only seen in if the muscles are incubated in  $\text{Ca}^{2+}$ - free

medium and with inhibitors of  $\text{Ca}^{2+}$  - dependent proteases. Otherwise, the  $\text{Ca}^{2+}$  - dependent pathway is triggered.<sup>43</sup>

The short – lived regulatory, cellular components, but also long – lived proteins that comprise the bulk of cells and myofibrillar proteins in fasting are digested by the proteasome proteolytic system.

The ATP – dependent proteolytic system is soluble and ubiquitin – requiring. The proteolytic unit of this system is a 26S proteasome, a large proteolytic complex that degrades proteins modified by the addition of polyubiquitin chain.<sup>44</sup> This conjugation of ubiquitin to proteins occurs in a series of steps involving E3 ubiquitin ligases, which are the key enzymes in this process. Notably, two E3s are expressed uniquely in muscle.<sup>45</sup>

### 9.3 Muscle protein breakdown inhibition

In a catabolic state, such as cancer cachexia, sepsis, diabetes, chronic renal failure, or in cases where nutritional supply or degree of contractile activity is not sufficient protein breakdown is accelerated.<sup>46</sup>

The regulation of proteolysis in muscles is not only important for amino acid homeostasis, but for the overall energy homeostasis as well. As amino acid generation is the first step in gluconeogenesis, it is not surprising that overall rate of protein degradation and synthesis in muscle are regulated by hormones that are also critical in energy homeostasis, notably insulin and cortisol.

Every proteolytic system can be inhibited by some inhibitor(s). The table 3 summarizes some of the inhibitors that influence different proteolytic processes.<sup>47</sup>

Treatments that influence the different proteolytic processes in incubated skeletal muscles

Treatments	Proteolytic Process	Actions
<b>Insulin, IGF-1, Amino Acids</b>	<i>Lysosomal</i>	<b>Suppress autophagic Vacuole formation</b>
<b>Weak bases (methylamine, Chloramines),</b>	<i>Lysosomal</i>	<b>Raise intralysosomal pH</b>
<b>Leupeptin , E64</b>	<i>Lysosomal</i>	<b>Inhibit cathepsins B, H, L</b>

Treatments	Proteolytic Process	Actions
Dinitrophenol + Glucose deprivation	<i>ATP – dependent</i>	Depletion of cellular ATP
Hemin	<i>ATP – dependent</i>	Inhibits high molecular weight

		proteases
Leupeptin, E64	<i>Ca<sup>2+</sup>-dependent</i>	Inhibit Ca <sup>2+</sup> -dependent proteases
Dantrolene		Decrease Ca <sup>2+</sup> release from sarcoplasmatic reticulum
Ca <sup>2+</sup> - ionophore		Activates by increasing Ca <sup>2+</sup> influx

Table 3: Proteolytic pathways inhibitors

Evidently, a broad specter of substances can play a role in protein breakdown inhibition. Most of them are proteolytic pathway specific. However, for inhibition of total protein breakdown, one can only use an inhibitor that is multipotent – inhibits all pathways. There are not many multipotent protein breakdown inhibitors. The two most researched are insulin and insulin like growth factor 1 (IGF – 1).

#### 9.3.1 Insulin

Insulin is famous for its regulatory function in glucose homeostasis, but one of its most important functions is to promote net accumulation of cell protein, especially in muscles.<sup>48</sup> The level of protein in muscles then determines the rate of hepatic gluconeogenesis. Insulin has several complementary anabolic actions on muscle:

- 1) It stimulates amino acid transport in to the tissue by an activation of the sodium – dependent transport system.<sup>49</sup>
- 2) It enhances protein synthesis by promoting the initiation of translation.<sup>50</sup>
- 3) Inhibits protein degradation<sup>51</sup>

Insulin and glucose (but not fatty acids or ketone bodies, although they are the preferred energy substrates for muscles) have additive effects in promoting the accumulation of amino acids in muscle. Without insulin and glucose, as in untreated diabetic patients, normal muscle growth cannot occur.

Although some authors argue that insulin does not inhibit myofibrillar protein degradation<sup>52</sup>, it is proved that insulin stimulates synthesis of myofibrillar proteins. **Error! Bookmark not defined.**

Anabolic effect of insulin is demonstrated in cardiac muscle and in liver as well.

Although findings by Goodman and Goldberg indicate that insulin is not affecting nonlysosomal pathway, Bennett et al and prove that insulin also inhibits proteasome in interaction with insulin – degrading enzyme.<sup>53</sup>

Such a holistic protein breakdown inhibitory effect makes insulin very appealing as a solution for controlling amino acids plasma level.

However, high dosage of insulin has to be followed by administration of considerable amount of glucose. This lengthy administration of high dosages of insulin and glucose is cumbersome and can lead to side effects such as fatty liver.

### 9.3.2 Insulin like growth factor 1 (IGF – 1)

IGF-1 and IGF-2 are polypeptides identified in serum showing activities similar to those of insulin, but they were not recognized by anti-insulin antibodies.

IGF – 1 is synthesized in liver and other cells in response to growth hormone. An infusion of recombinant IGF – 1 does help diabetic rats overcome growth failure, without influencing the blood glucose levels.<sup>54</sup> These strong anabolic effects of IGF – 1 without significant hypoglycemic actions in vivo, gives this polypeptide a great potential to counter the excessive proteolysis seen in various catabolic situations.

Interestingly, Insulin and IGF – 1 seem to have additional effect at physiological concentrations (100 ng/l). At high concentrations (1000 ng/l), this additivity disappear, as both polypeptides compete for the same receptors.

IGF – 1 does have great potential for the studied anti – cancer, arginine depletion therapy. The only, but serious constraint is that in the scientific society as well as in the regulatory bodies, IGF – 1 is perceived as a substance which generates tumor growth. It would be difficult get the permission to administer IGF – 1 to the cancer patients.

## 10 APPENDIX IV: THE GUT AND ARGININE METABOLISM

### 10.1 The gut functions and amino acids

The gut's primary function in the digestion of food and the assimilation of nutrients is well studied. Its role in protection, through a continuous exposure to dietary toxins and pathogens is also well known. Approximately 70% of the total immune cell population resides in the gut.<sup>55</sup> The gut also has a crucial role in coordinating the activity of all organs involved in the food ingestion. This information transfer is possible thanks to an extensive and intrinsic neural system located in the gut.

It is not surprising that the gut utilizes a huge amount of matter, notably amino acids, and fuels a substantial quantity of energy for fulfilling these demanding functions.

A lot of research has shown that amino acids play a critical role in the gut intermediary metabolism, secretion, absorptive and protective function. Some remarkable results underlined a special function of the gut in the amino acid metabolism:

1. The Young's group at the M.I.T. has proved that significant quantities of amino acids 'disappeared' between the gut and the peripheral circulation. This but also other works have shown that the first-pass metabolism of dietary acids is largely a function of the metabolic activity of the gut and not this of the liver.<sup>56</sup> It is documented that at least 25% of the total protein intake is used in the splanchnic bed, the large majority by the

gut. Even more interestingly, there is a big variation in degree to which different amino acids are utilized, so that the gut substantially modifies the mixture of amino acids available to the organism.

Indeed, virtually all the dietary glutamate is metabolized in first pass in addition to the well-established utilization of arterial glutamine by intestinal mucosa.<sup>57</sup>

2. The second striking finding is that in the fed state, there is simultaneous utilization of both arterial and luminal essential amino acids by the intestinal tissues.<sup>58</sup> The majority, in quantitative terms, of amino acids for metabolism in portal – drained viscera comes from the arterial side! This finding raises the important issues with regard to the regulation of amino acid transport from the mesenteric artery through the basolateral membrane of the enterocytes.
3. If the dietary protein intake is restricted, the gut still utilizes the amino acid by itself. The usage of the arterial amino acid can be lowered, but the gut growth continues even if the nutritional conditions are such that the muscle growth is stopped.

## 10.2 The gut, arginine, proline and lactate

As presented in the figure 7, arginine is a semi – essential amino acid in the healthy adults, as it can be endogenously synthesized in the enterocytes from the non – essential amino acids proline and glutamine/glutamate.

There are two pathways for the endogenous, de novo, arginine production. The one is from glutamine/glutamate and the other one is from proline. The both pathways lead to the  $\Delta^1$  – L – Pyrroline – 5 Carboxylate (see figure 9). The crucial enzymes in this process of arginine synthesis are the proline oxidase (indicated as the #7 in the figure 9), the P5C synthase (indicated as the # 5) and the ornithine amino transferase (indicated as the #6 in the figure 9).



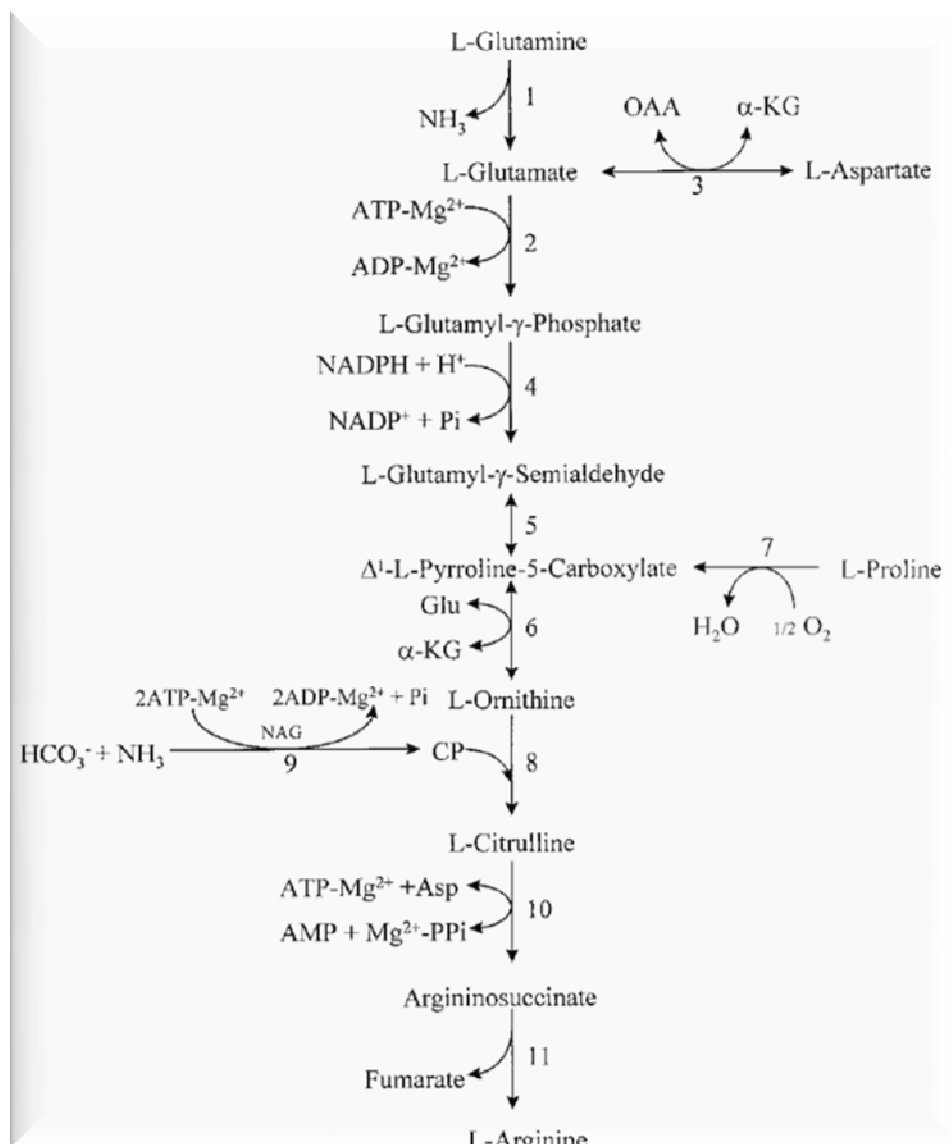


Figure 9: Endogenous synthesis of arginine in enterocytes

Once wrongly reported to be present only in liver and kidney, proline oxydase is found to have in enterocytes 10- and 6 – fold higher activity compared to the liver and kidney respectively. Several authors indicate that both metabolic and enzymological evidence prove that the small intestine is the major source of circulating citrulline for endogenous synthesis of arginine in neonates and adults.<sup>59</sup>

In a series of effective experiments WU G. et al.<sup>60</sup> have demonstrated that lactate inhibits citrulline and arginine in enterocytes of the 14 – day – old pigs.

These experiments have elucidated why an elevated plasma concentration of lactate led to a severe deficiency of circulating arginine and citrulline (plasma level below detection limit) in a

human infant.<sup>61</sup> A deficiency of arginine with elevated lactate concentration is documented in adults too<sup>62</sup>.

### 10.3 The gut, arginine, glutamine and pyridoxal-5'-phosphate

The figure 6 clearly indicates that the central molecule in the arginine synthesis from the proline and glutamine/glutamate is the  $\Delta^1$ -L-Pyrroline-5-Carboxylate. It is either synthesized directly from the proline, or from glutamine, through a series of metabolic steps.

It is logical though, and the experiments by several authors proved this hypothesis, that lactate alone is not enough for the inhibition for the endogenous arginine production.

The production of the  $\Delta^1$ -L-Pyrroline-5-Carboxylate from glutamine/glutamate should be blocked as well. One possibility was to inhibit the P5C synthase, which is indicated as #5 in the figure 7, by pyridoxal-5'-phosphate.

Another possibility was to inhibit conversion of  $\Delta^1$ -L-Pyrroline-5-Carboxylate into ornithine by gabaculine. The gabaculine inhibits the enzyme Ornithine amino transferase (OAT), which is indicated as the #6 in the figure 9.

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## **ACKNOWLEDGMENT**

I am honored to thank Prof. Dr. P. M. Montavon, Head of Small Animal Surgery, Vetsuisse Faculty, University of Zurich and Prof. Dr. C. Evans, Maurice Müller Professor of Orthopedic Surgery, Director of the Center for Advanced Orthopedic Studies, Harvard Medical School, for refereeing / co – refereeing this dissertation; to Dr. Sci. Slobodan Tepic, Head of Cancer Treatment International and Kyon AG, Zürich, for his teaching, guidance and support during this thesis writing and throughout my whole career, to Prof. Dr. A. Pospischil, Head of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, and his team for performing pathology and pathohistology, to Nicolas Späth, DVM for being a great collaborator, to Pawel Pyk, PhD and Darek Mochnacki, dipl. ing., for their assistance with amino acid analyzer and to my beloved family for their support and patience.



## **CURRICULUM VITAE**

Name: **Goran Cvetkovic, DVM, MBA**

Date of birth: **08.11.1972**

Place of birth: **Smederevo, Serbia**

Nationality: **Serbian**

### **Education**

1991-1997	Study of Veterinary Medicine at the Veterinary Faculty, University of Belgrade, Serbia
2005-2007	Study at Graduate School of Business Administration, Horgen, Switzerland, with BS of Business Administration
2007-2009	Study at R.H.Smith School of Business, University of Maryland, USA, with MS of Business Administration
2009-2010	Doctoral Student (Inaugural Dissertation), Clinic of Small Animal Surgery, Vetsuisse Faculty University of Zurich